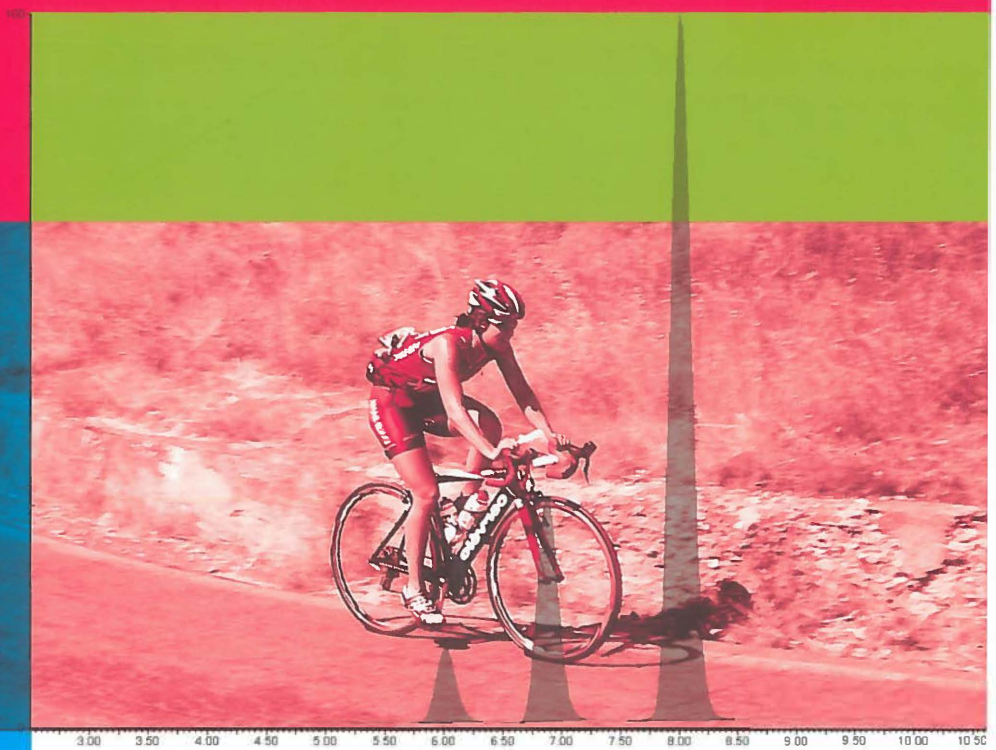
NEUROENDOCRINE BIOMARKERS IN CLINICAL CHEMISTRY

METHOD DEVELOPMENT AND CLINICAL VALIDATION OF AUTOMATED LC-MS/MS METHODS



2010

Helma de Jong

NEUROENDOCRINE BIOMARKERS IN CLINICAL CHEMISTRY: METHOD DEVELOPMENT AND CLINICAL VALIDATION OF AUTOMATED LC-MS/MS METHODS

Wilhelmina HA de Jong

STELLINGEN

behorende bij het proefschrift

NEUROENDOCRINE BIOMARKERS IN CLINICAL CHEMISTRY
METHOD DEVELOPEMENT AND CLINICAL VALIDATION OF AUTOMATED LC-MS/MS METHODS

1) Koppeling en automatisering van de monstervoorbewerking vergroot de mogelijkheden van LC-MS/MS. *(dit proefschrift)*

2) Toepassing van XLC-MS/MS leidt tot verbetering van de juistheid, precisie, gevoeligheid en efficiëntie bij de analyse van biomarkers met een laag molecuul gewicht.. *(dit proefschrift)*

3) Met het beschikbaar komen van massaspectrometrische analyses voor routinematig gebruik, dienen immunoassays ontraden te worden voor de analyse van biomarkers, waarvan bekend is dat ze gestoord worden door moleculen met een vergelijkbare structuur.

4) Bij de aanvraag en beoordeling van laboratoriumtesten van gedeconjugeerde catecholaminen en metanefrinen dient rekening te worden gehouden met de invloed van catecholamine-bevattende voedingsmiddelen. *(dit proefschrift)*

5) Omdat vrij in plasma circulerende metanefrinen niet worden beïnvloed door dieetsamenstelling heeft deze test de voorkeur boven urine metanefrinen en catecholaminen bij de biochemische diagnose van feochromocytoom. *(dit proefschrift)*

6) Met het beschikbaar zijn van een betrouwbare bepaling voor vrij 3-MT in plasma zijn er nieuwe mogelijkheden voor de biochemische diagnostiek van het paraganglioom en neuroblastoom. *(dit proefschrift)*

7) Polymorfisme in de serotonine transporter is van invloed op de concentratie vrij in plasma circulerend serotonine in patiënten met serotonine producerende carcinoid tumoren.

8) De effecten van overmatige ananas, banaan en walnoot consumptie op sociaal functioneren binnen een laboratoriumafdeling dient, net zoals de effecten hiervan op biochemische resultaten, niet onderschat te worden.

9) Met XLC-MS/MS gaat alles automatisch maar niets vanzelf.

10) Hoewel halverwege de berg het uitzicht ook mooi is, blijft de top toch altijd het doel.

11) Sportieve inspanning leidt tot geestelijke ontspanning.

12) Positief zijn zit in je bloed.

13) Ik praat niet te snel, je luistert te langzaam. (Loesje)

Helma de Jong
Groningen, 7 april 2010

de Jong, W.H.A.
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NEUROENDOCRINE BIOMARKERS IN CLINICAL CHEMISTRY: METHOD DEVELOPMENT AND CLINICAL VALIDATION OF AUTOMATED LC-MS/MS METHODS

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Beoordelingscommissie:	Prof. dr. T.P. Links Prof. dr. R.J.P. Bischoff Prof. dr. M.A. Blankenstein

Paranimfen

Drs. I. van den Broek
M. Jelies

CONTENTS

List of Abbreviations

AADC	aromatic acid decarboxylase
ACE	automatic cartridge exchanger
CID	collision induced dissociation
CLSI	clinical and laboratory standards institute
COMT	catechol-O-methyltransferase
CV	variation coefficient
ECD	electrochemical detection
EDTA	ethylenediaminetetraacetic acid
GC-MS	gas chromatography with mass spectrometric detection
5-HIAA	5-hydroxyindole acetic acid
HILIC	hydrophilic interaction chromatography
3-HK	3-hydroxykynurenine
HPD	high pressure dispenser
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
IDO	indoleamine 2,3-dioxygenase
KYN	kynurenine
LC	liquid chromatography
LC-MS/MS	liquid chromatography with tandem mass spectrometric detection
LLE	liquid liquid extraction
LLOD	lower limit of detection
LLOQ	lower limit of quantification
MAO-A	monoamine oxidase A
MN	metanephrine
MNs	metanephrines
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
3-MT	3-methoxytyramine
NMN	normetanephrine

PBA	phenyl boronic acid
PFP	pentafluorophenyl
PRS	propylsulphonic acid
R^2	correlation coefficient
RAM	restricted access media
SD	standard deviation
SERT	serotonin transporter
SPE	solid phase extraction
SSRI	selective serotonin reuptake inhibitor
SULT1A3	sulfotransferase 1A3
TDO	tryptophan-2,3-dioxygenase
TRP	tryptophan
UPLC	ultra performance liquid chromatography
WCX	weak cation exchange
XLC-MS/MS	on-line solid phase extraction coupled to liquid chromatography with tandem mass spectrometry

Chapter 1

Introduction and aims of the thesis

Introduction and aims of the thesis

There is a wealth of new insights in the (patho)physiological background and metabolic pathways involved in the commence of diseases. This results in a steady increase of newly discovered biomarkers and new applications for long-known compounds.
In clinical biochemistry knowledge about biochemical pathways is crucial in order to reveal which compounds are clinically relevant to quantify. Also, knowledge about analytical techniques needed for the quantification of compounds of interest is essential. Analytical insights are continuously evolving as a consequence of the development of new, or the improvement of existing techniques. Currently, tandem mass spectrometric detection (MS/MS) connected to liquid chromatography (LC) is emerging in analytical and clinical chemistry. The number of publications on the application of LC-MS/MS in laboratory diagnosis has grown significantly in the last ten years. Introduction of this technique has remarkably improved sensitivity, specificity and accuracy when compared to non-mass spectrometric chromatographic methods. Therefore this detection technique currently is considered as the most promising method for the analysis of low molecular weight biomarkers and the profiling of related compounds. An obvious and important advantage of LC-MS/MS is that it can easily be automated which is important for reduction of variation and analysis time in the laboratory[1]. Therefore, the development and clinical validation of biochemical mass spectrometric assays currently plays an important role in clinical biochemistry. Nowadays, the application of technically advanced equipment for such analyses is evolving. At the Department of Laboratory Medicine of the University Medical Center Groningen sample preparation (i.e. solid phase eXtraction) is on-line coupled with LC-MS/MS. Shortly this technique is referred to as XLC-MS/MS (Fig. 1).

The aim of the research described in this thesis was to develop and validate mass spectrometric methods for the quantification of biomarkers used in the biochemical diagnosis of neuroendocrine tumors. An additional aim was to determine unambiguously the influence of certain pre-analytical factors, such as diet, on concentrations of neuroendocrine markers in plasma and urine as obtained by the analytically well-performing mass spectrometric methods. Quantification of neuroendocrine analytes is an analytical challenge because of their chemical instability and low concentrations ranges. For this purpose we designed different analytical strategies, all using the XLC-MS/MS platform. In **chapter 2** the broader perspective of the evolvement of LC-MS/MS in neuroendocrinology is overviewed from relevant articles published in the last decade and indexed on PubMed. LC-MS/MS applications are compared with more conventional techniques such as immunoassays, high performance liquid chromatography (HPLC) and gas chromatography with mass spectrometric detection (GC-MS). Furthermore, automation

of sample preparation and its potential to improve the performance of the laborious error-prone manual sample pretreatment is highlightened.

Catecholamine metabolism (part I of this thesis).

Catecholamines are biogenic amines with metanephrines (O-methylated catecholamines) as diagnostic important metabolites (Fig. 2). In order to study the metabolic processes of catecholamines, reliable techniques for their quantification in body fluids are essential[2].

The aim of **chapter 3** is to describe the method development and clinical validation of the most important marker for pheochromocytoma diagnostics, i.e. plasma free metanephrines. A pheochromocytoma is a neuroendocrine catecholamine-producing tumor, usually located in the adrenal medulla, arising from chromaffin cell tissues. Cornerstone of pheochromocytoma diagnosis is the demonstration of a catecholamine excess. Plasma free metanephrines have demonstrated to be the analytes in this metabolic pathway with the highest diagnostic sensitivity and specificity[3]. Since no well-performing analytical method has been available for world-wide application of plasma free metanephrines, the development and clinical validation of such a method would be a major contribution to improve quality of the biochemical diagnosis in the routine clinical chemical setting.

The development of an accurate interference-free method for the measurement of plasma free metanephrines, allowed examination of the impact of pre-analytical exogenous factors, such as physiological influences, medication or food products[3-6], on the outcome of the test.

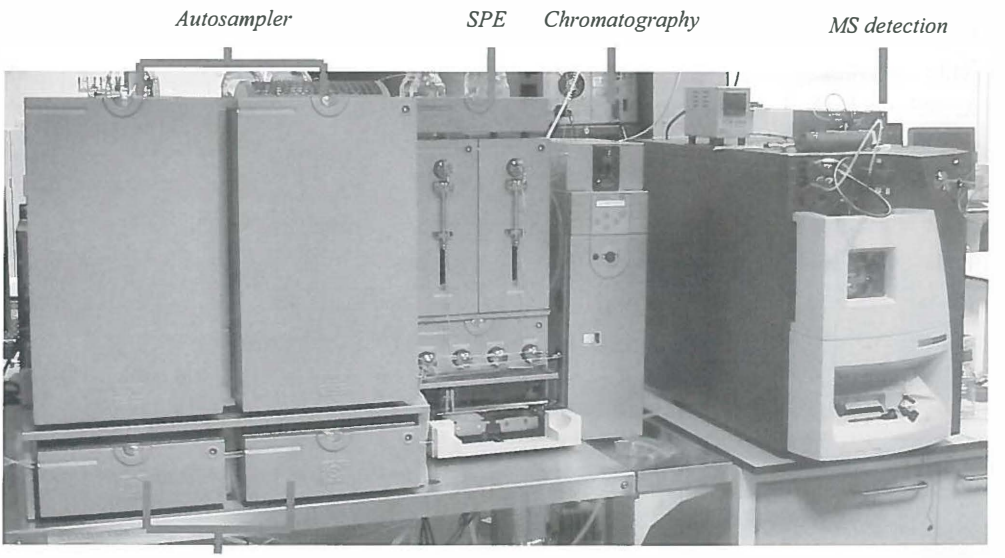

Figure 1. The XLC-MS/MS apparatus.
Abbreviations: *SPE*: solid phase extraction (sample pretreatment); *MS*: mass spectrometry.

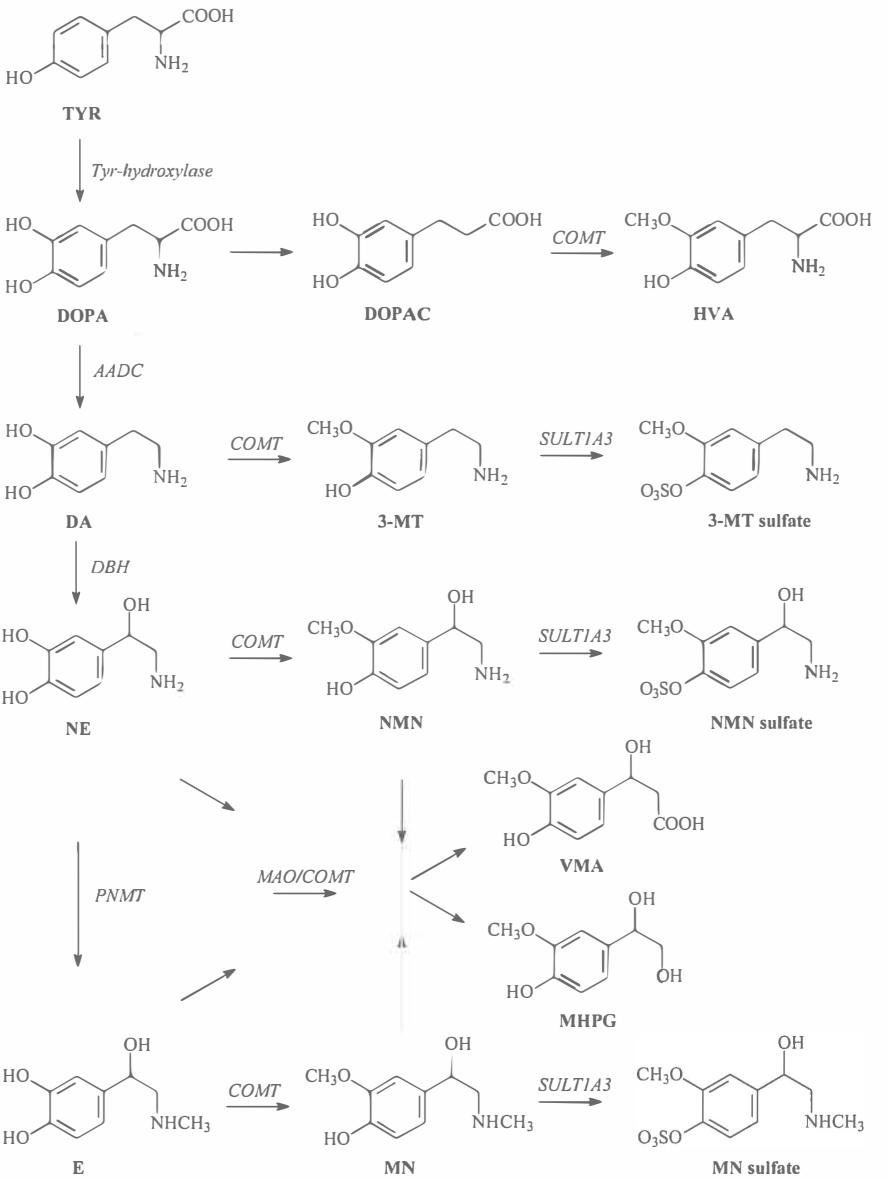

Figure 2: Schematic overview of the catecholamine metabolism.
Abbreviations: TYR: tyrosine, DOPA: 3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: homovanillic acid, DA: dopamine, 3-MT: 3-methoxytyramine, NE: norepinephrine, NMN: normetanephrine, VMA: vanillylmandelic acid, MHPG: 3-Methoxy-4-hydroxyphenylglycol, E: epinephrine, MN: metanephrine, COMT: catechol-O-methyltransferase, AADC: aromatic acid decarboxylase, SULT1A3: sulfotransferase1A3, PNMT: Phenylethanolamine N-methyltransferase, MAO: monamine oxidase.

The aim of **chapter 4** is therefore to describe the influence of consuming catecholamines, present in food products, on the free and deconjugated (free + conjugated) plasma and urinary metanephrine levels in healthy volunteers subjected to catecholamine-rich or catecholamine–poor dietary restrictions. In addition, the influence of sitting or supine blood sampling posture on plasma metanephrines outcomes is described.

Methods for the measurement of the parent catecholamines themselves are laborious and analytically challenging. Therefore, the aim of **chapter 5** is to describe the development and clinical validation of a sensitive and specific automated XLC-MS/MS method for urinary catecholamine analysis in order to replace the commonly applied laborious manual sample preparation methods coupled to interference-prone detection techniques. In addition, **chapter 6** investigates the pre-analytical influence of consuming a catecholamine-rich diet on the free and deconjugated catecholamine concentrations as described for metanephrines in chapter 4.

Tryptophan metabolism (part II of this thesis).

The essential amino acid tryptophan is the precursor of several biological active compounds. For instance, tryptophan is the precursor of indoles, such as serotonin and melatonin (Fig. 3). The aim of **chapter 7** is to describe the development and clinical validation of a sensitive and specific XLC-MS/MS method for the quantification of urinary 5-hydroxyindole acetic acid (5-HIAA), which is the end-product of serotonin metabolism in urine. The automated, but interference-prone HPLC technique used before, employing fluorometric detection, required optimization to continue in providing accurate results. The biochemical analysis of urinary 5-HIAA is essential, since it is the golden standard for follow-up of carcinoid patients[7,8]. In carcinoid patients upregulation of tryptophan metabolism (Fig. 3) results in increased synthesis of serotonin and it major metabolite 5-HIAA[9-11].

Carcinoids arise from enterochromaffin cells, located in the gastro-intestinal tract and appear most frequently in the midgut and to a lesser extent in fore- and hindgut. Urinary serotonin has been implicated in the diagnosis of unusual foregut carcinoid tumors[7,10,12,13]. The interference-prone HPLC fluorometric method used before, suffers from extensive manual sample pretreatment. Aim of **part A** of **chapter 8** is therefore to describe the improvement of urinary serotonin quantification by the development and clinical validation of an automated XLC-MS/MS method and to establish new reference intervals for a healthy population. An additional goal in this chapter is to make the method applicable for free serotonin in plasma as well and to demonstrate the clinical relevance of the free fraction by comparing results from carcinoid patients with healthy subjects. The free fraction is the active form which potentially is hazardous and may cause specific serotonin-excess related symptoms. Tightly controlled reuptake and metabolism mechanisms in the body keep free

serotonin at low levels. Due to those low concentrations this analyte has always been difficult to measure[9]. Clinical utility of free serotonin has been proposed in the development of selective serotonin reuptake inhibitor (SSRI) -derived osteoporosis[14,15]. This issue and the suggestion to measure free serotonin are addressed in **part B** of **chapter 8** in the form of a letter responding to the article dealing with this question[15].

Another route in the tryptophan metabolic pathway is the kynurenine pathway (Fig. 3), induced by the enzyme indole-2,3-dioxygenase (IDO). No high-throughput methods are available for the simultaneous detection of tryptophan and the kynurenine metabolites. Therefore the aim of **chapter 9** is to describe the development and clinical validation of the high-throughput simultaneous measurement of tryptophan, kynurenine and 3-hydroxykynurenine with XLC-MS/MS. Additionally, chapter 9 describes the within-day biological variation of these three compounds. Knowledge about this pre-analytical factor is important for the interpretation of results. Monitoring of tryptophan levels could be considered routine care for carcinoid patients since long-term augmentation of the serotonin biosynthesis in carcinoid patients can result in serious reduction of the free tryptophan body pool. In addition, the favoring of the serotonin pathway can cause deficiencies in the alternative IDO-initiated tryptophan metabolic pathway (Fig. 3) towards kynurenines[16,17]. Combined analysis of tryptophan and kynurenines offers the possibility to discover new insights about the role of tryptophan degradation in neuropsychiatric and oncologic diseases.

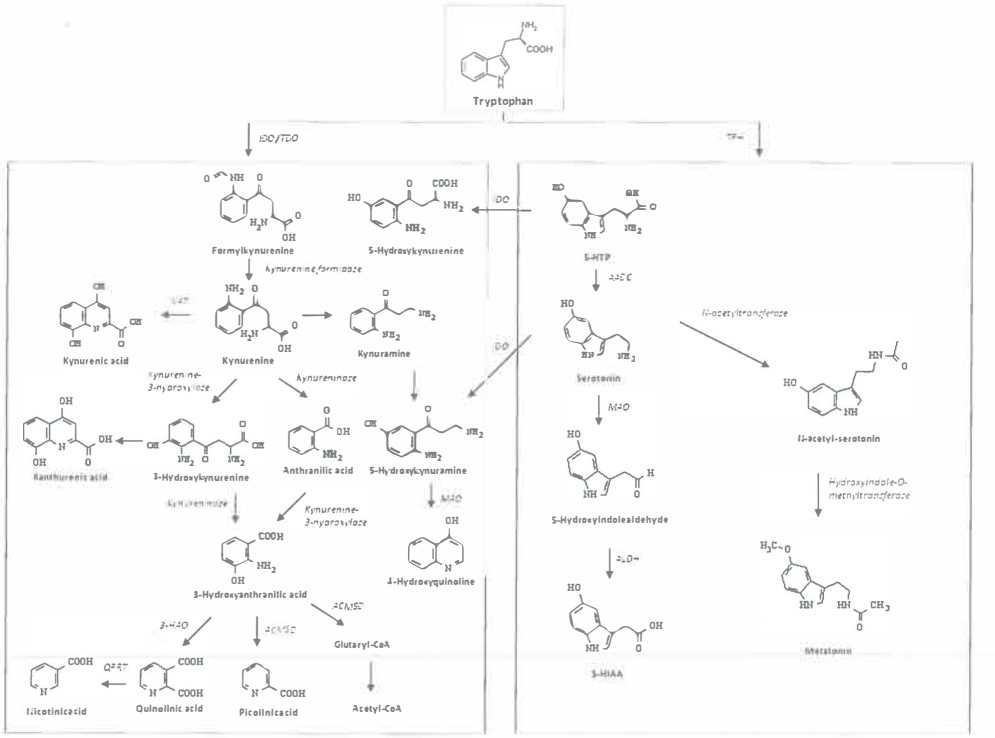

Figure 3. Schematic overview of the tryptophan metabolism.
Abbreviations: *IDO*: indole-2,3-dioxygenase; *TDO*: tryptophan-2,3-dioxygenase; *TPH*: tryptophanhydroxylase; *5-HTP*: 5-hydroxytryptophan; *AADC*: aromatic acid decarboxylase; *KAT*: kynurenine aminotransferase; *MAO*: monoamine oxidase; *ALDH*: aldehyde dehydrogenase; *3-HAO*: 3-hydroxyanthranilic acid oxygenase; *ACMSD*: aminocarboxymuconate semialdehyde decarboxylase; *QPRT*: quinolinate phosphoribosyltransferase; *CoA*: coenzyme A; *5-HIAA*: 5-hydroxyindole acetic acid.

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Chapter 2

Current Status and Future Developments of LC-MS/MS in Clinical Chemistry for Quantification of Neurochemical Biomarkers

Wilhelmina HA de Jong
Elisabeth GE de Vries
Ido P Kema

Submitted for publication

Abstract

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is rapidly gaining ground in special clinical chemistry laboratories. It significantly increases the analytic chemical potential, especially in the field of low molecular weight biomarker analysis. This review overviews current LC-MS/MS methods used for the quantification of biogenic amines and their metabolites. New possibilities offered by this technique are illustrated by recently developed assays for biogenic amines.

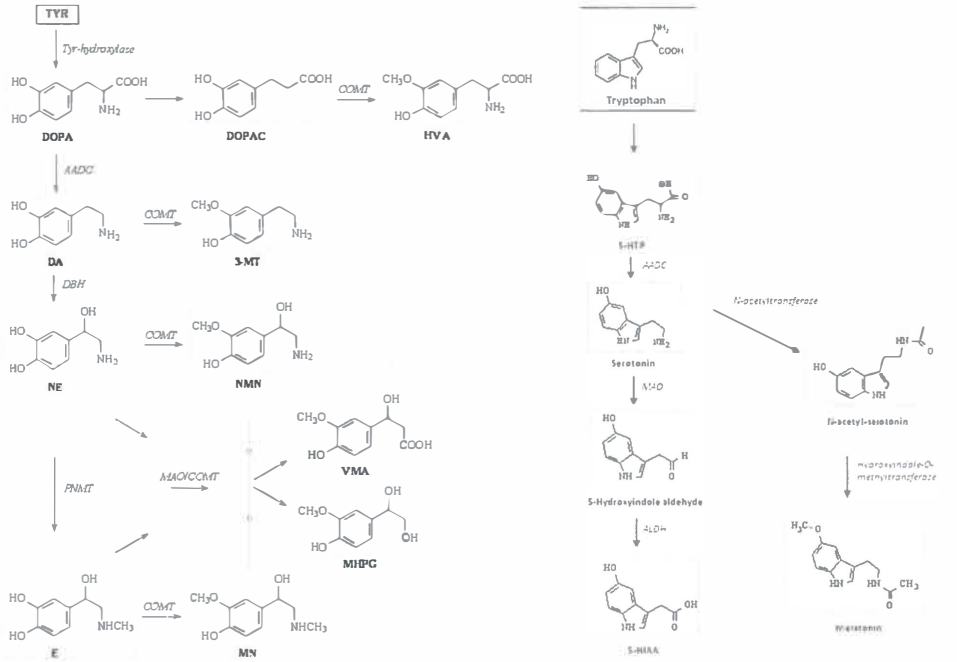
Major shortcomings of conventional chromatographic techniques, such as labor-intensive sample preparation, long analysis times and often the relatively low specificity, are circumvented by using LC-MS/MS. In addition, LC-MS/MS has broad analyte compatibility and high analytical performance. In the last five years introduction of LC-MS/MS in routine diagnostics has resulted in improved assays for diagnosis and follow-up of neuroendocrine tumors characterized by the secretion of biogenic amines. Due to their labile nature and low concentration ranges biogenic amines require extensive and careful sample preparation. Introduction of new sophisticated techniques such as selective sorbents adsorption is evolving. This enables not only more specific analyte selection, but also automation of the complicated clean-up procedure. Automated sample clean-up can be directly coupled to LC-MS/MS, which facilitates reproducible and efficient handling of the growing number of samples to be analyzed in laboratories.

Introduction

High performance liquid chromatography coupled to tandem mass spectrometric detection (LC-MS/MS) is becoming an indispensible technique in the special chemistry laboratories in clinical chemistry as it greatly increases sensitivity and specificity of test results. Applying this technique will result in improved biochemical diagnosis of endocrine disorders, and opens new roads to gain insight in pathophysiological processes. This review gives an overview of the current methods and analytical developments for quantification of biogenic amines and their metabolites as used for the biochemical diagnosis and follow-up of neuroendocrine tumors. Analytical principles and clinical implications of the developed methods are emphasized. Sample preparation techniques that have been described for the analysis of compounds of interest are accentuated.

Clinical background

Biogenic amines is a term referring to catecholamines, serotonin and histamine, which are neurotransmitters and hormones. They are synthesized from amino acids: catecholamines from tyrosine, serotonin from tryptophan and histamine form histidine. Biogenic amines function throughout the body, both in the central nervous system and in the periphery. Disorders affecting their metabolism or action can have devastating effects on homeostasis of the human body[1]. In clinical chemistry quantification of biogenic amines is mainly applied for diagnosis of neuroendocrine tumors such as pheochromocytoma and carcinoids. A pheochromocytoma is a neuroendocrine tumor, originating from chromaffin cell tissues. It is usually located in the adrenal medulla. Pheochromocytoma is characterized by the excessive secretion of catecholamines, which can cause symptoms such as sustained or intermittent hypertension, sweating, tachycardia, and palpitations[2]. Diagnosis depends on demonstration of the excessive catecholamine production, usually achieved by analysis of plasma and urinary free catecholamines and catecholamine metabolites[3,4]. Highly sensitive and specific biochemical tests are required for correct clinical chemical diagnosis, avoidance of false-negative results, and follow-up of patients. The 3-O-methylated metabolites, i.e. metanephrine (MN) from epinephrine, normetanephrine (NMN) from norepinephrine and 3-methoxytyramine (3-MT) from dopamine (Fig. 1A), have recently proven to be particularly useful. Measurements of these metanephrines in plasma or urine are currently recommended for the diagnosis of pheochromocytoma due to their highest diagnostic sensitivity and specificity[5]. Because metanephrines occur in very low concentrations (nmol/L), high-performing analytical techniques are required for their reproducible quantification.



A B

Figure 1: Schematic overview of the catecholamine and serotonin synthesis and metabolism.
Shown are the quantitative most important compounds in the catecholamine and serotonin pathway. *A*: Catecholamine synthesis and metabolism. *B*: Serotonin synthesis and metabolism.
Abbreviations: TYR: tyrosine, DOPA: 3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenyl-acetic acid, HVA: homovanillic acid, DA: dopamine, 3-MT: 3-methoxytyramine, NE: norepinephrine, NMN: normetanephrine, VMA: vanillylmandelic acid, MHPG: 3-Methoxy-4-hydroxyphenylglycol, E: epinephrine, MN: metanephrine, COMT: catechol-O-methyltransferase, AADC: aromatic acid decarboxylase, PNMT: phenylethanolamine N-methyltransferase, MAO: monamine oxidase, TPH: tryptophan hydroxylase; 5-HTTP: 5-hydroxytryptophan; ALDH: aldehyde dehydrogenase; ALDR: aldehyde reductase; 5-HIAA: 5-hydroxyindole acetic acid.

Carcinoids are gastrointestinal neuroendocrine tumors that appear most frequently in the midgut and to a lesser extent in fore- and hindguts[6,7]. When metastasized, they may give rise to excessive serotonin production with concomitant symptoms of the carcinoid syndrome, including flushing, diarrhea, right-sided hart disease and bronchoconstriction. Increased serotonin synthesis from its precursor tryptophan, may reduce tryptophan levels available for other metabolic pathway routes. Urinary 5-HIAA is the final breakdown product of serotonin and the current standard for diagnosis and follow-up of carcinoid patients (Fig. 1B)[6]. Serotonin stored in platelets is the most sensitive marker for the detection of minor serotonin overproduction and therefore important for the diagnosis of (metastatic) carcinoids[8]. The measurement of urinary serotonin has been implicated in the diagnosis of foregut carcinoid tumors even though they produce serotonin much less frequently than midgut carcinoids[9]. By augmentation of the serotonin pathway, the synthesis of compounds derived from other tryptophan metabolic routes (i.e. the kynurenine pathway with niacine as end-product) is reduced[10].

Methods of measurement

For the measurement biogenic amines in biological matrices LC-MS/MS needs to compete with conventional high performance liquid chromatography (HPLC) with ultraviolet, fluorescence or electrochemical detection and to a lesser extend gas chromatographic methods and immunoassays[11].

2.1 Conventional HPLC

The most commonly technique used for the quantification and profiling of biogenic amines is reversed phase high performance liquid chromatography (HPLC) with ultraviolet, fluorescence or electrochemical detection[6,12-15]. Characteristic features of most biogenic amines are their oxidation vulnerability and their native fluorescence properties, which explain the long history of conventional HPLC detection methods. The design of conventional HPLC assays is straightforward, which enables in-house development and improvement of methods. Technical skills from lab technicians and extensive data interpretation are however required, while practicability and throughput are generally low[16]. Other disadvantages are the extensive sample preparation required for appropriate sample clean-up and the long chromatographic separation times to achieve complete chromatographic separation of the target analytes and the frequently occurring matrix interferences. Specificity and sensitivity are variable, dependent on the analyte and the detection technique[17-19].

2.2 GC-MS

Gas chromatography with mass spectrometric detection (GC-MS) is a valuable tool in clinical chemistry, owing to its high identification and separation power combined with high sensitivity[18]. It is used for quantification of several biogenic amines, i.e. for the catecholamine metabolites homovanillic acid (HVA) and vanillyl mandelic acid (VMA)[20] and metanephrines[21,22]. GC-MS, as HPLC, enables profiling of related compounds in one assay, which allows an overview of metabolically related compounds. GC-MS requires the analytes to be stable, volatile, and amenable to the ionization techniques (i.e. electron impact or chemical ionization). Most compounds need derivatization to meet these requirements, and similar to HPLC methods GC-MS needs extensive time-consuming sample preparation with larger sample volumes. In addition long chromatographic run times are used to obtain sufficient separation of compounds, especially for profiling purposes. Practicability of GC-MS is poor, and consequently its application has remained limited to a smaller number of specialized endocrinology laboratories[18,23].

2.3 IMMUNOASSAYS

Today, immunoassays represent the predominant technology used for routine analysis of endogenous and exogenous steroids and other hormones. For the quantification of biogenic amines immunoassays are also applied, although to a lesser extent. Major strengths of immunoassays are the potential of full automation, random access and high throughput combined with practicability and the relative little technical expertise required to operate. Furthermore, immunoassays are known to have the highest analytical sensitivity of all quantification techniques[24]. However, limitations with respect to analytical specificity and accuracy, cross-reactivity, nonspecific binding or interferences by other endogenous or exogenous compounds are well recognized, due to matrix effects. Furthermore different assays are needed for related compounds and the concentration range is narrow[23-27]. The lack of internal standards in immunoassays reduces confidence in reported values. Results from different assays are therefore difficult to compare, which complicates inter-laboratory comparison. On the other hand inter- and intra-assay variability of the commercially obtained assays cause difficulties for patient follow-up over time and longitudinal studies[23-27]. It is complicated and expensive to develop immunoassays in-house, which implicates dependency of the diagnostic industry[19,23-25]. From an analytical perspective, however, other techniques are better suited for the measurement of biogenic amines than immunoassays. This is also demonstrated by the fact that no random access immunoassays have been described in the last five years for biogenic amines except for metanephrines[28-30].

2.4 LC-MS/MS

In pharmaceutical industries and toxicology laboratories LC-MS/MS is the method of choice for the development and the measurement of drugs[18]. For clinical chemical analyses LC-MS/MS is rapidly emerging, as demonstrated by the amount of 171 articles published concerning this technique in the journal Clinical Chemistry during the last 5 years. It offers advantages such as high analytical sensitivity, specificity and accuracy and is applicable to a broad selection of compounds, especially for the diagnosis of aberrations within the endocrine system, such as biogenic amines[31-36], but also steroids[37], thyroid hormones[38], thyroglobulin[39] and vitamin D[40-42]. Furthermore LC-MS/MS enables the use of sophisticated sample pretreatment techniques and automation of the whole process by on-line coupling of the separate techniques.

LC-MS/MS combines the physical separation capabilities of HPLC with the high analytical sensitivity and specificity of mass spectrometric detection. In recent years, LC-MS/MS equipment has been improved in performance. Due to its superior specificity, shorter runtimes and less laborious sample preparation LC-MS/MS methods replace more and more of the above described HPLC, GC-MS and immunoassay techniques[17-19]. LC–MS/MS has, just as HPLC and GC-MS, the advantage that several compounds can be measured simultaneously[43]. Profiling allows evaluation of metabolic pathways which might simplify diagnosis and is more cost-effective. A difficulty with LC-MS/MS is the interindividual variation in body fluids composition, which can result in alteration of ionization efficiency. These matrix effects are compound-specific and need to be evaluated for[23].

LC-MS/MS analysis of biogenic amines

Recent publications show that the introduction of LC-MS/MS methods in special clinical chemistry labs has important implications for the analysis of biogenic amines and their respective metabolites (Table 1), such as catecholamines[31-34] and indoles[34-36]. Application of this technique generally results in increased specificity, reproducibility, shorter runtimes and improved sample handling by simpler sample preparation techniques, compared to conventional HPLC analysis[17-19,44].

HPLC combined with non-mass spectrometric detection can be prone to analytical interferences that complicate the measurement of the low-concentrated biogenic amines. Medications such as acetaminophen and methenamine, for example, can influence the analysis of catecholamines and metanephrines[45,46]. Immunoassays on the other hand may suffer from cross-reactivity and lack an internal standard affecting the accuracy of results[47]. LC-MS/MS based analyses, do not suffer from these drawbacks, due to their mass specific

detection. This is exemplified in the measurement of plasma free metanephrines for the diagnosis of pheochromocytoma[48-50]. The first assays of plasma free metanephrines made use of HPLC with electrochemical detection[51]. This necessitated the use of extensive sample clean-up and long chromatographic run times. The introduction of mass spectrometry led to more specific methods with simpler although manual sample clean-up[52]. However, this method, which utilized SPE extraction, was unable to reach the sufficient analytical sensitivity to reach the required detection limit. In addition, long chromatographic run times were needed to achieve separation of the metanephrines and potential interferences. The introduction of on-line solid phase extraction coupled to LC-MS/MS (XLC-MS/MS) resulted shortened chromatographic run times and automation of sample preparation[48]. In addition, specificity was increased by application of weak cation exchange SPE with sample preconcentration due to on-line elution into the LC-MS/MS and hydrophilic interaction chromatography (HILIC). The application of HILIC resulted in fast chromatography and small peaks with good signal-to-noise ratio, although short dwell times were required. Specificity was also increased by the use of additional qualifiers measured in the tandem mass spectrometer. The combination of on-line SPE, HILIC and tandem mass spectrometry demonstrated to be a powerful combination to reach the required detection limits of <1 nmol/L for MN and NMN and 3-MT. The opportunity to quantify 3-MT was an additional advantage, because this biomarker has the potential to detect dopamine-producing tumors such as paragangliomas and neuroblastomas[53]. The precise role of 3-MT however, needs further investigation. Urinary deconjugated metanephrines have been analyzed with LC-MS/MS for a longer period of time[54], since these markers occur in higher concentration ranges and require less sensitive assays. Although diagnostic sensitivity and specificity of metanephrines in urine are less than in plasma[5], urinary metanephrines are also applied for the diagnosis of pheochromocytoma. The same holds true for the catecholamine metabolite homovanillic acid (HVA)[33,24]. Automated solid phase extraction can also be applied for this marker. Described methods use hydrophobic interaction[33,54] or SPE can be omitted[34]. Reversed phase chromatography results in shorter runtimes of 3 versus 30 min per sample[33] and detection limits are below 0.05 mg/L[33,24]. HVA nowadays mainly is used as a marker for the diagnosis of dopamine metabolite producing neuroblastomas[55]. For the diagnosis of pheochromocytoma, plasma free metanephrines are recommended since they have higher diagnostic sensitivity and specificity[5].

LC-MS/MS methods also have been developed for the measurement of low catecholamine concentrations in urine[32]. Catecholamines assist in the diagnosis of neuroendocrine catecholamine-producing tumors, such as pheochromocytoma and neuroblastoma, in

addition to metanephrines and HVA described above[5,56] and are therefore still commonly applied. Because of the low physiologic concentrations of catecholamines and the tendency of the catechol group to be oxidized, their measurement is complicated. For sample clean-up liquid-liquid extraction (LLE) was applied after specific complexation of the catecholgroup with phenyl boronic acid, similar to extraction previously described for HPLC with electrochemical detection[57]. Chromatographic separation was achieved by column material containing a polar group imbedded in a hydrophobic chain, which is suitable for the separation of basic polar compounds without ion-pairing reagents[32] and is an obvious advantage when using mass spectrometric detection.

Serotonin in blood is mainly stored in platelets. Free serotonin occurs in low concentrations in plasma because of active reuptake and fast metabolism which complicates detection with most conventional techniques. Platelet serotonin is specifically measured for the detection of carcinoid tumors that secrete little serotonin. With LC-MS/MS it is possible to measure accurate and reproducible serotonin both in platelet-rich and platelet-poor plasma[58,59]. For this purpose protein precipitation, using acetonitrile, combined with chromatography based on strong cation exchange and reversed-phase interaction, was used with a total run time of 6 minutes[59] and a detection limit of 5 nmol/L. Solid phase extraction based on weak cation exchange and HILIC, comparable to the method described for plasma metanephrines, resulted in the same analysis time with detection limits even below 1 nmol/L[58]. Because serotonin is involved in several physiologic processes in, such as hormonal regulation in the gastro-intestinal tract, neurotransmission in the central nervous system and mental disorders[60-62]. Therefore, being able to measure the free form of serotonin in lower concentrations might open new doors. For example in the follow-up of depressed patients[63] or for the elucidation of the recently proposed role of serotonin in the development of osteoporosis[64]. One of the most commonly analyzed compound in the serotonin metabolic pathway is 5-hydroxyindole acetic acid, which is used for the follow-up of carcinoid patients. Due to its relatively high levels in urine, routine measurements are often performed without complications. However, for LC-MS/MS sample pretreatment is warranted and interferences are likely to occur. Several LC-MS/MS methods have been described for this marker. 5-HIAA can be measured without sample preparation following direct injection and reversed phase chromatography[34]. Also on-line extraction methods based on hydrophobic interaction have been reported[35,65]. On-line SPE demonstrated low imprecision (<5%) and short analysis time of 5 min[35] or even 3 min[65].

Table 1. Analysis characteristics of biogenic amines measurable by LC-MS/MS in clinical chemistry

Compound	Detection Limit	Conventional technique	LC-MS/MS sample pretreatment	Reference
Biogenic amines				
Catecholamines	5 ng/mL	HPLC	SPE	31
	2.5 µg/L		LLE	32
	1 µg/mL		SPE	66
	0.01 µmol/L		Complexation, SPE	67
	10-30 pg		LC	68
Metanephrines	0.10 nmol/L	HPLC	SPE	48
	0.13 nmol/L		SPE	49
	0.09-0.17 nmol/L		Protein precipitation	50
	10 µg/L		SPE	54
	1 µg/mL		SPE	66
	0.005 µmol/L		Complexation, SPE	67
5-HIAA	0.015 mg/L	HPLC	No pretreatment	34
	0.13 µmol/L		SPE	35
	4 µmol/L		SPE	65
Serotonin	0.9 nmol/L	HPLC	SPE	58
	1.5 nmol/L		Protein precipitation	59
	20 pg		LC	68
Tryptophan/ kynurenines	1-30 nmol/L	HPLC/ GC-MS	SPE	36
	3 nmol/L		Protein precipitation	69
HVA	0.01 mg/L	GC	SPE	33
	0.05 mg/L		No pretreatment	34
	1 µg/mL		SPE	66

Kynurenines, other metabolites from the serotonin precursor tryptophan, that are implicated in immune responses as result of indoleamine-2,3-dioxygenase (IDO) activity, are also target compounds of LC-MS/MS. Newest developments for these compounds reducing analysis time and manual sample pretreatment are on-line SPE (strong cation exchange) coupled to LC-MS/MS for the measurement of 3-hyroxykynurenine next to tryptophan and kynurenine[36]. Protein precipitation coupled to capillary LC-MS/MS[69] enables the simultaneous analysis of kynurenic acid, tryptophan and kynurenine. Chromatography was based on reversed-phase interaction and detection limits were in the low nmolar range[36,69].
Profiling of several neurotransmitters in one run has been an important issue in the last years. For instance catecholamines and metabolites can be analyzed in the same run using

reversed phase chromatography[66,67]. SPE after formation of phenyl boronic acid complexes coupled to LC-MS/MS enables combined measurement of catecholamines and metanephrines[67]. Direct plasma injection without sample preparation and internal-surface reversed phase chromatography with time-of-flight mass spectrometric detection allowed quantification of nine catecholamine metabolic pathway compounds, including catecholamines, metanephrines and HVA[66]. This profiling might help in the diagnosis of neuroblastoma. However, sensitivity is less than for specific biogenic amines or metabolite assays mentioned before. Also, compounds of the serotonin and catecholamine metabolic pathways can be analyzed together[34,68]. This potentially offers the possibility to screen for several kinds of neuroendocrine tumors with one analysis. Urinary 5-HIAA, VMA and HVA are measured following reversed phase chromatography without sample pretreatment[34]. The neurotransmitters dopamine, epinephrine, norepinephrine, 3,4-dihydroxybenxylamine, salsolinol, serotonin and gamma-aminobutyric acid can simultaneously be detected in aqueous standard solutions following reversed phase chromatography without sample pretreatment and with detection limits below 30 pg[68]. No results were shown for biological matrices however.

Sample pretreatment

Sample preparation is often required for LC-MS/MS, since biological matrices are not directly compatible with this technique. Clean-up protocols for plasma and urine samples are generally time consuming and the most laborious and error-sensible parts of analysis protocols. Currently, LLE can only be performed off-line in laborious procedures, while SPE, immunoaffinity and restricted access media (RAM) can be automated and on-line coupled to the LC-MS/MS. Sometimes, only deproteinization is used to prevent protein denaturation in the analysis system which damages the column or contaminates the detector[70]. A major risk, when using this procedure, however, is ion suppression in the mass spectrometer, which is caused by matrix interferences and harms reproducibility and sensitivity[71]. The goals of sample preparation are therefore to remove matrix constituents such as proteins and lipids and to isolate and pre-concentrate the analytes of interest for improvement of selectivity, sensitivity, accuracy and reproducibility of the analysis[70,72]. Sample preparation traditionally consists of protein precipitation, LLE and SPE. A possible disadvantage of simple protein precipitation is that the remaining sample still contains interfering components. In addition, no sample preconcentration or oxidationprotection occur. Concerning the other sample clean-up techniques, SPE is often preferred over LLE because it minimizes or eliminates some of the drawbacks of LLE, such as the need to use

large amounts of organic solvent. Moreover this procedure can be easily automated. SPE is the most common sample clean-up technique in clinical, pharmaceutical, environmental, food and industrial chemistry and is based on the affinity of the analyte for a specific solid phase sorbent[70,73].

Despite the risk of occurrence of interferences, ion suppression and oxidation, protein precipitation is also applied for biogenic amines[34,66,68]. Because of their (positively) charged state, which is the result of presence of the amine group, biogenic amines can be easily isolated by cation exchange SPE sorbents. This requires however, an acidic environment that also prevents oxidation of these compounds[74]. Metabolites of biogenic amines are often more stable and less oxidation sensitive, which enables storage and sample-clean-up conditions[75].

The typical SPE steps, i.e. conditioning of the sorbent, sample application, sample wash and elution of the target analytes can be easily automated with systems available by several companies with the use of any commercial cartridge with the specific sorbent materials[73]. Sample clean-up can be coupled directly to LC-MS/MS, with the additional advantage that samples are preconcentrated which increases sensitivity, as demonstrated for the measurement of plasma free metanephrines[48]. Automation typically improves accuracy, increases the sample throughput, decreases the costs per sample and improves the productivity of personnel and instruments[19,76].

Developements in sample pretreatment

To increase the application of SPE in LC-MS/MS, sophisticated kinds of selective sorbents are being developed, like immunoaffinity sorbents and restricted access media (RAM). They are a valuable addition to traditional hydrophobic interaction and ion-exchange principals[77], because specificity of this conventional SPE is not always sufficient for the target analytes. In addition, recovery might be increased by the use of new sorbents.

Automation

Automated sample preparation for LC-MS/MS can be performed in various degrees, i.e. using robotic liquid handlers for shortening of pipetting time[78], column switching[79] or on-line extraction systems with transfer to autosampler vials[80]. The direct injection of plasma using an on-line extraction method coupled to LC, where the whole analytical procedure takes place in a closed, automated system, is a promising method for automation and is referred to as XLC-MS/MS. Since the sample preparation step is embedded into the chromatographic separation most of the manual sample preparation time is eliminated, while the amount of required sample is reduced and preconcentration of the sample occurs.

In addition, the samples are less exposed to the environment, which is especially important for biogenic amines that are susceptible to light and oxygen. To what extent binding protein interactions are broken is unknown, which is a potential disadvantage for some compounds[76,81-83]. Examples are Prospekt or Symbiosis systems from Spark Holland (Emmen, The Netherlands), OSP-2 from Merck (Darmstadt, Germany) and ASPEC XL from Gilson, Inc. (Middleton, WI). Automated on-line SPE has shown to effectively lower detection limits and analysis times, and increase reproducibility and precision of assays for the measurement of biogenic amines[35,36,48,76]. In addition to this analytical improvement, by providing reproducible results automation also increases clinical reliability.

RESTRICTED ACCESS MEDIA

The term RAM was introduced in the early 90s[84] and refers to its first function, which is the separation of macromolecules (mostly proteins) from low-molecular compounds by limiting the accessibility to the sorbent for the larger molecules (restricted access). This size-exclusion of macromolecules is achieved by physical (pore size) (Fig. 2A) or chemical (bonded phase of protein or polymer networks) diffusion barriers (Fig. 2B). Consequently macromolecules can interact only with the outer surface of the particle support. This outer surface consists usually of hydrophilic groups, which is not favourable for protein binding, resulting in washing away of proteins. Secondly, specific sample clean-up takes place with matrix components that are small enough to penetrate into the pores or are able to pass the chemical barriers. At the specific binding sites within the porous materials, sorbent materials are present similar to regular SPE sorbents[72,84,85]. Naturally, the RAM can be specifically classified based on the chemical characteristics of the surfaces[86].

The use of RAM has been successfully applied for direct extraction and concentration of hydrophobic low molecular mass analytes from biological fluids with high proteins levels (i.e. plasma, blood, urine, saliva). The materials are suitable for the on-line coupling methods and during recent years, this on-line sample extraction process has been widely used for the fast analysis of a large variety of compounds in various biological matrices[73]. For the analysis of urinary catecholamines RAM has also been applied coupled to conventional HPLC-ECD and proved to be highly accurate in a broad concentration range from physiological to pathological levels[87]. Therefore RAM seems to be a promising sample pretreatment technique for biogenic amines.

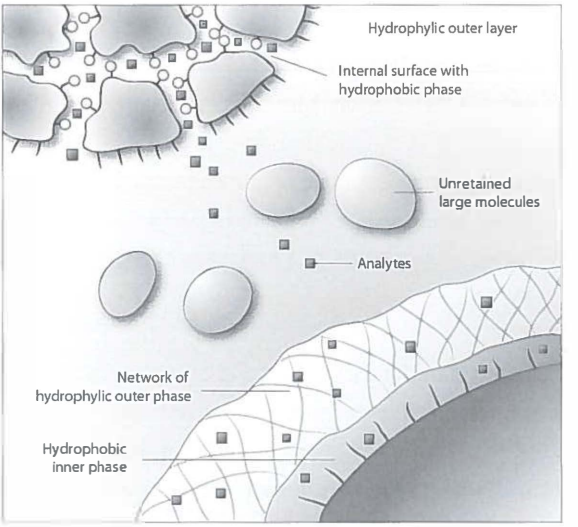


Figure 2: Principle of restricted access media (RAM) for sample cleanup.
Separation of macromolecules (mostly proteins) from low-molecular compounds is achieved by limiting the accessibility to the sorbent for the larger molecules (restricted access).
A: Size-exclusion of macromolecules is achieved by a physical (pore size) barrier. Macromolecules can interact only with the outer surface (represented by the purple blocks) of the particle support consisting of hydrophilic groups. Because this binding principle is unfavourable for large molecules, they are not retained but washed away. Low-molecular analytes that are small enough to penetrate through the barriers are subjected to further sample clean-up. The analytes interact with the specific binding sites consisting of regular solid phase extraction sorbents (such as hydrophobic material represented by the squares and circles attached to the outer layer) within the porous materials. After washing steps have been applied, target analytes can be eluted using specific solvents that disrupt the interaction. B: Size-exclusion of macromolecules is achieved by a chemical (bonded phase of polymer networks) diffusion barrier. Macromolecules can interact only with the outer surface (represented by the green outer layer) of the particle support consisting of hydrophilic polymer networks, because they are unable to penetrate through the polymer network. Low-molecular analytes that can penetrate through the first layer, reach the second (purple) layer. The analytes interact with the specific binding sites of this surface consisting of regular solid phase extraction sorbents (such as hydrophobic material). After washing steps have been applied, target analytes can be eluted using specific solvents that disrupt the interaction.

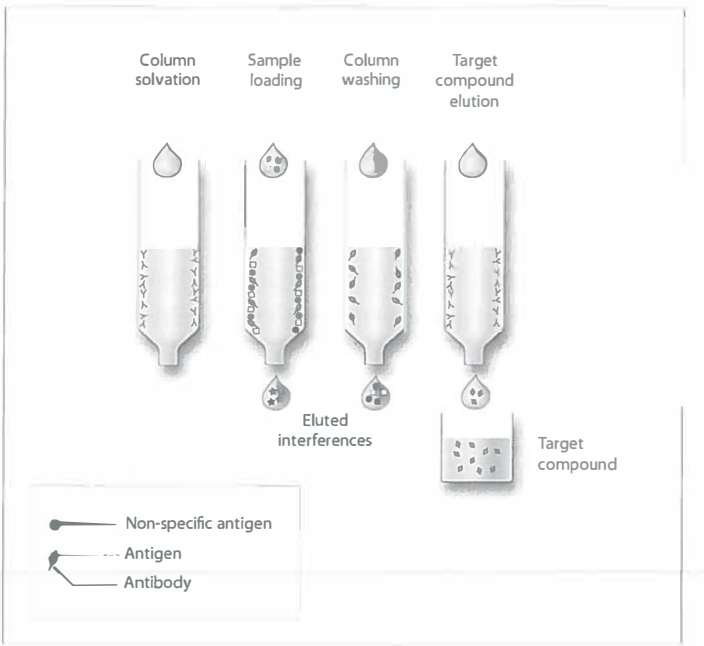


Figure 3: Immunoaffinity principle.
Immunoaffinity is a specific form of solid phase extraction with sorbents based on the affinity between antibody and antigen (analyte). Specific antibody-antigen binding in the sample cleanup column is achieved by interaction of the antigen with the sorbent-bound antibody. First, the column material is solvated to activate the antibody. Subsequently, the sample is loaded on the column in such a way that interaction can occur. Thereafter non-specific matrix compounds are washed away. In the elution step, the specific antibody-antigen binding is disrupted in order to release the analytes for further analyses and detection.

IMMUNOAFFINITY SORBENTS

Immunoaffinity sorbents are based on the affinity between antibody and antigen (analyte), caused by molecular recognition (Fig. 3). The first immunoaffinity sorbents have been described for large molecules because of the availability of the antibodies for such molecules. Obtaining selective antibodies for low-molecular mass compounds is more difficult and the development of immunochemical methods in the solid-phase extraction field targeting low-molecular-mass analytes is recent[70]. Extensive studies demonstrating the advantages of coupling immunoaffinity extraction directly to HPLC for smaller compounds have been performed for the determination of estrogens and nortestosterone in plasma and urine by using column switching[88,89]. This was a promising onset to use immunoaffinity for the measurement and profiling of steroids. It is clear that immunosorbents have potential to

be useful in case of difficult sample matrices such as plasma and urine. In the last few years there has been considerable interest in the pharmaceutical and environmental fields both for off-line and on-line immunoaffinity sample clean-up, although most applications continue to originate from the same research groups[90-93]. No immunoaffinity sample clean-up applications for biogenic amines have been described yet, which might be related to the availability of appropriate antibodies for these compounds. The immunoaffinity clean-up principle, however, is promising for neuroendocrine assays that need improvement regarding detection limits and reproducibility which cannot be obtained with conventional techniques. For the analysis of testosterone, immunoaffinity clean-up has shown to be effective[94], which demonstrates potential of this technique for other endocrine hormones.

Conclusions

LC-MS/MS is becoming an indispensible tool for low-molecular weight biomarker quantification also in the field of special clinical chemistry. It overcomes drawbacks of conventional techniques, such as long analysis times and imprecision, has a broad analyte compatibility and high analytical performance. It enables more sensitive and specific measurement of biogenic amines and metabolites, and the routine quantification of biomarkers in low concentration ranges. Specific sample preparation techniques and automation of the complete procedure, allow reproducible and efficient handling of larger sample series. For biogenic amines, implementation of specific on-line SPE techniques leads to decreased detection limits, increased specificity and more accurate and sensitive quantification. In addition, laboratory efficiency is improved due to shorter analysis time and a reduction of manual sample preparation. As such the technique proves to be a valuable tool for the investigation of the biochemical aspects of neuroendocrine diseases.

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PART I

Catecholamine metabolism

Chapter 3

Plasma Free Metanephrine Measurement Using Automated Online Solid-Phase Extraction HPLC–Tandem Mass Spectrometry

Wilhelmina HA de Jong
Kendon S Graham
Jan C van der Molen
Thera P Links
Michael R Morris
H Alec Ross
Elisabeth GE de Vries
Ido P Kema

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Abstract

Quantification of plasma free metanephrine (MN) and normetanephrine (NMN) is considered to be the most accurate test for the clinical chemical diagnosis of pheochromocytoma and follow-up of pheochromocytoma patients. Current methods involve laborious, time-consuming, offline sample preparation, coupled with relatively nonspecific detection. Our aim was to develop a rapid, sensitive, and highly selective automated method for plasma free MNs in the nanomole per liter range. We used online solid-phase extraction coupled with HPLC-tandem mass spectrometric detection (XLC-MS/MS). Fifty microliters plasma equivalent was prepurified by automated online solid-phase extraction, using weak cation exchange cartridges. Chromatographic separation of the analytes and deuterated analogs was achieved by hydrophilic interaction chromatography. Mass spectrometric detection was performed in the multiple reaction monitoring mode using a quadrupole tandem mass spectrometer in positive electrospray ionization mode. Total run-time including sample cleanup was 8 min. Intra- and interassay analytical variation (CV) varied from 2.0% to 4.7% and 1.6% to 13.5%, respectively, whereas biological intra- and interday variation ranged from 9.4% to 45.0% and 8.4% to 23.2%. Linearity in the 0 to 20 nmol/L calibration range was excellent (R^2>0.99). For all compounds, recoveries ranged from 74.5% to 99.6%, and detection limits were <0.10 nmol/L. Reference intervals for 120 healthy adults were 0.07 to 0.33 nmol/L (MN), 0.23 to 1.07 nmol/L (NMN), and <0.17 nmol/L (3-methoxytyramine). This automated high-throughput XLC-MS/MS method for the measurement of plasma free MNs is precise and linear, with short analysis time and low variable costs. The method is attractive for routine diagnosis of pheochromocytoma because of its high analytical sensitivity, the analytical power of MS/MS, and the high diagnostic accuracy of free MNs.

Introduction

A characteristic of pheochromocytomas is the excessive secretion of catecholamines, which cause typical symptoms such as sustained or intermittent hypertension, sweating, tachycardia, and palpitations[1-4]. The prevalence of pheochromocytoma in patients with hypertension is estimated to be 0.1% to 0.5%, with 20% to 75% of these cases remaining undetected before death[5,6]. Highly sensitive and specific biochemical tests are required for correct clinical chemical diagnosis, avoidance of false-negative results, and follow-up of patients.

The main metabolic routes of catecholamines are oxidative deamination by monoamine oxidase (EC 1.4.3.4) and *O*-methylation by catechol-*O*-methyltransferase (EC 2.1.1.6) or both. Metanephrines (MNs) are the products of enzymatic conversion of catecholamines by catechol-*O*-methyltransferase: 3-methoxymetanephrine (3-MT) from dopamine, normetanephrine (NMN) from norepinephrine (NE), and MN from epinephrine[1-4].

Traditional biochemical tests for the diagnosis of pheochromocytoma are based on the presence of several compounds in the catecholamine metabolic pathway. Some of these analyses have limitations with respect to analytical or diagnostic sensitivity or specificity and analysis time[1-4]. Recent studies have highlighted the higher diagnostic accuracy of plasma free MN measurements over tests that quantify catecholamines. This difference is a consequence of constant leakage of catecholamines from vesicular stores (in tumor cells) into the cytoplasm, where they are continuously metabolized to MNs[7,8]. Although diagnostic specificity may be increased, false-positive results may still occur owing to interference from prescribed medications or diet[7,9,10,11].

Because plasma MNs occur in low concentrations in a complex matrix and their chemical characteristics are not unique, developing an assay for these compounds is an analytical challenge. Currently, HPLC using electrochemical detection (ECD) is the method of choice[7,12,13]. However, data interpretation may be complicated because of coeluting interference, and improvements can be made in reducing labor-intensive sample cleanup and total analysis time. Alternatively, rapid measurement of plasma free MN can be achieved by the use of immunoassays[14,15]; however, cross-reactivity and nonspecific binding may lead to erroneous results. In addition, the lack of an internal standard in such assays reduces confidence in reported values. GC-MS methods address some concerns regarding sensitivity and specificity. Nevertheless, sample preparation procedures for these methods involve derivatization and extraction, which are laborious and time-consuming. Given the limitations of existing analytical techniques, the need for an alternative, simplified method of analysis is clear.

An LC-MS/MS method using offline solid-phase extraction (SPE)[16] appears to be superior to GC-MS methodologies in terms of both sensitivity and sample throughput. However, considerable improvements could be made to the selectivity of the SPE procedure and sensitivity of the analysis. Furthermore, as sample preparation has been highlighted as a major contributor to the total analysis time observed in previous methods, automation of the extraction process and coupling it directly to the chromatographic system would be expected to lead to significant benefits in sample throughput and precision. This process is termed online SPE–liquid chromatography (XLC).

This study describes a high-throughput, sensitive, specific, and automated XLC-MS/MS method that enables simultaneous extraction, concentration, separation, and mass-selective detection of plasma free MNs for the diagnosis of pheochromocytoma.

Materials and Methods

Reagents

We obtained HPLC-grade acetonitrile and methanol from Rathburn Chemicals; ammonium formate 99.995+% and disodium EDTA from Sigma-Aldrich; formic acid 98% to 100% ultrapure from BDH Laboratory Supplies; and sodium hydroxide (NaOH), hydrochloric acid, and sodium metabisulfite ($Na_2S_2O_5$) from Merck KGaA. Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure.

We purchased *D,L*-metanephrine-HCl, *D,L*-normetanephrine-HCl, and *D,L*-3-methoxytyramine-HCl from Sigma-Aldrich, the deuterated internal standards α,α,β-d_3-metanephrine-HCl and α,α,β,β-d_4-3-methoxytyramine-HCl from Cambridge Isotopes, and α,α,β-d_3-normetanephrine-HCl from Medical Isotopes.

Stock solutions and samples

We prepared stock solutions in 0.1 mol/L HCl. Stock solutions were serially diluted and used to form calibrators and low, medium, and high quality-control samples in pooled plasma via enrichment. The concentration range of the calibrators was from physiological levels (0 to 1 nmol/L) to approximately 20 nmol/L for all analytes.

Plasma samples from patients with confirmed pheochromocytoma for whom these tests were ordered (as established by the routinely used GC-MS method for urinary fractionated MNs and pathology reports) came from our own hospital (University Medical Center, Groningen). We collected blood samples by venipuncture, with the patient in a sitting position, in 10-mL Vacutainer Tubes (Becton Dickinson) containing K_2EDTA solution as

anticoagulant. After centrifugation, we transferred the plasma to glass tubes containing 5 mg $Na_2S_2O_5$ as a preservative, and samples were stored at –20 °C until analysis.

Before analysis, we mixed aliquots of plasma samples (500 μL) with 100 μL internal standard stock solution (4.95 nmol/L in diluted acetic acid) and diluted them with 400 μL water. We placed sample vials in the autosampler, and 100 μL of each sample (equivalent to 50 μL of plasma) was injected. Required sample volume for automatic injection can be scaled down to 50 μL by using microliter pickup injection mode.

ANALYSIS AND QUANTIFICATION

Instrumentation

We used a Spark Holland Symbiosis® online SPE system for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger), a solvent delivery unit (2 high-pressure dispensers), and an HPLC pump, as shown in Fig. 1. The automated cartridge exchanger module contains 2 connectable 6-way valves and an SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and cleanup. The integrated HPLC pump was a binary high-pressure gradient pump.

We used Oasis® weak cation exchange 10 by 1 mm SPE cartridges (Waters Corp.) for sample extraction and performed HPLC by use of an Atlantis HILIC Silica column (particle size 3 μm, 2.1 mm internal diameter by 50 mm; Waters). Column temperature was controlled with a Mistral Column Oven (Spark Holland). Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

Online SPE

We performed online SPE following a similar method described by Kema et al.[17]. The Symbiosis system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted. The analytes were eluted directly on the analytical column, as is schematically shown in Fig. 1. In the first step, the SPE cartridge was automatically located in the left clamp for conditioning and equilibration (Fig. 1A). The sample was passed on to the extraction cartridge (Fig. 1B) using water as the loading solvent, and wash solvents were applied (Fig. 1C). The extraction cartridge was then automatically transferred to the right clamp for elution of the analytes directly on the analytical column by passing the chromatographic mobile phase

through the cartridge for 2 min (Fig. 1D). After elution, chromatographic separation on the analytical column occurred and the right clamp, containing the cartridge, was flushed (Fig. 1E). Processing of subsequent plasma samples was carried out in parallel.

Liquid chromatography

The binary gradient system consisted of 100 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid (eluent A) and acetonitrile (eluent B). Gradient elution was performed according to the following elution program: 0 min, 5% A, 95% B; 6 min, 20% A, 80% B; 6–7 min, 20% A, 80% B; 7.5 min, 5% A, 95% B; reequilibration from 7.5 to 8 min with 5% A, 95% B. Gradients applied were linear; flow rate was 0.400 mL/min. Column temperature was kept at 20 °C.

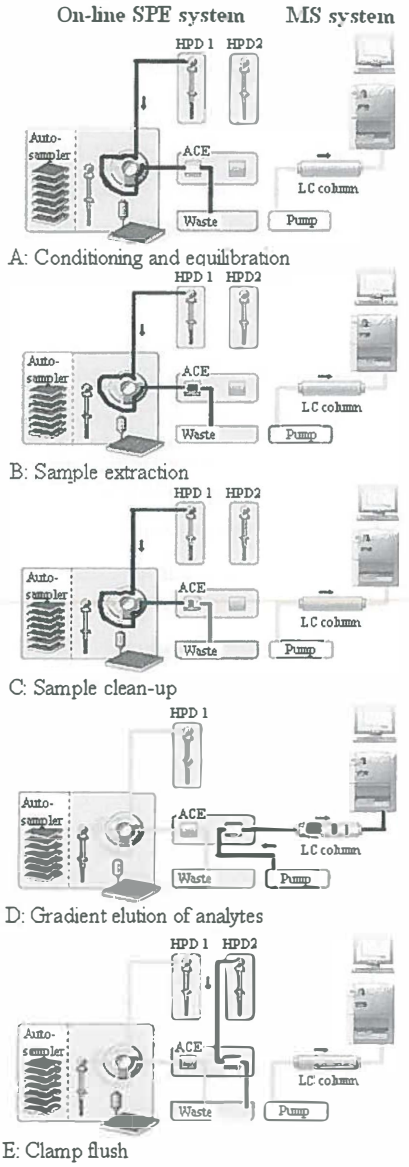
Mass spectrometry

In positive ionization mode, MN, NMN, and 3-MT were protonated to produce ions at the form $[M+H]^+$. These ions are known to undergo a facile loss of water in the ion source, and the ion source conditions were optimized for these resulting ions of the form $[M+H-H_2O]^+$: MN, *m/z* 180; NMN, *m/z* 166, and 3-MT, *m/z* 151, as described by Lagerstedt et al.[16]. On collision-induced dissociation, these precursor ions produced characteristic product ions of *m/z* 148, *m/z* 134, and *m/z* 119, respectively. We developed a multiple reaction monitoring (MRM) method using a dwell time of 40 ms and an interchannel delay of 10 ms. Recently, the use of additional MRM transitions for absolute confirmation of the presence of a compound in an analytical method has been proposed (e.g., EU directive 2002/657/EC). For this reason, we used mass transitions *m/z* 198→180 (MN), 184→134 (NMN), and 151→91 (3-MT) as qualifiers.

Quality control and method validation

Selectivity

We verified the identities of sample MN, NMN, and 3-MT peaks by analysis of the compound specific mass spectra after addition of calibrator (standard addition).



A: Conditioning and equilibration

A1: New cartridge	From tray to left clamp
A2: Conditioning	Acetonitrile (1 mL at 2 mL/min)
A3: Equilibration	Water (2 mL at 2 mL/min)

B: Sample extraction

B1: Injection	Start loopfill with sample
B2: Extraction	Sample loading using water (500 µL at 500 µL/min)

C: Sample clean-up

C1: Wash cartridge	Water (1 mL at 2.5 mL/min)
C2: Wash cartridge	Ammoniumformatebuffer 10 mmol/L pH 3 (1 mL at 2 mL/min)

D: Gradient elution of analytes

D1: Transfer cartridge	From left to right clamp
D2: Elution	Elution of analytes from cartridge to LC column using LC gradient (1.0 min)
D3: New cartridge	From tray to left clamp

E: Clamp flush

E1: Clamp flush	Water (2 mL at 1 mL/min)
E2: Clamp flush	Acetonitrile (2 mL at 2 mL/min)
E3: Move cartridge	From right clamp to tray

Figure 1. Schematic representation of the Symbiosis XLC-MS/MS.
A: conditioning and activation of the SPE cartridge in the left clamp. *B:* sample extraction after filling of the autosampler loop. *C:* sample cleanup. *D:* right clamp gradient elution of analytes with LC mobile phase, directly followed by chromatographic separation and mass spectrometric detection. *E:* cartridge and clamp cleanup.
Abbreviations: *SPE*: solid phase extraction; *HPD*: high-pressure dispenser; *LC*: liquid chromatography; *ACE*: automatic cartridge exchanger, *MS/MS*: tandem mass spectrometry.

Detection limits

For plasma, we determined detection limits [limit of detection (LOD)] and quantification limits by injecting serially diluted samples containing MN, NMN, and 3-MT. LOD was defined as the injected amount that produced a signal-to-noise ratio of 3. Limit of quantification was defined as the injected amount that produced a signal-to-noise ratio of 10. We estimated the percentage of carryover between sequential analyses performed on new SPE cartridges by alternating injections of blanks and plasma samples with high concentrations of MNs.

Linearity and imprecision

We plotted the ratios of analyte peak area to internal standard peak area against MNs at 8 concentrations in the intervals 0.26 to 18.21 nmol/L for MN, 0.82 to 18.21 nmol/L for NMN, and 0.58 to 19.93 nmol/L for 3-MT. On 20 different days, we prepared and measured fresh calibration lines. The lines were calculated by use of Excel software and least-squares linear regression. We applied the CLSI EP-6P protocol[18] to test the linearity of the method. The dilutional linearity of the assay was performed in duplicate by serial dilution of enriched plasma samples with water.

We determined intra- and interassay variation by use of 3 pooled samples with MNs in low, medium, and high concentrations and obtained intraassay imprecision from 20 replicates measured in a single series and interassay imprecision from 20 different assays over a 3-week period.

Recovery

We estimated mean relative recoveries by the addition of MNs to plasma in low, medium, and high concentrations and measured recoveries in 6 replicates of these samples by using 2 cartridges placed in series.

Method comparison

Heparin-EGTA plasma samples measured previously, as described by Lenders et al.[13] using HPLC-ECD, were reanalyzed with the new XLC-MS/MS method. Samples were stored at –20 °C for 1 year. We applied Passing–Bablok regression analysis (using EP Evaluator, recommended by the CLSI)[19] for method comparison.

Stability

Samples with low, medium, and high concentrations of added MNs were measured in triplicate after different storage conditions. The first set was assayed immediately and served as reference point; other sets were stored at 10 °C (autosampler temperature) and 4

°C for 16, 24, and 48 h and 7 days and at room temperature for 24 h. The remaining samples were frozen at –20 °C, and stability was investigated after 1 to 3 freeze-thaw cycles.

Biological variation, reference values, and patient samples

We determined biological intra- and interday variation by analyzing plasma obtained from 10 healthy individuals in sitting position (5 men, 5 women, age range 20 to 56 years, median age 35.0 years), at 5 times during 1 day (09:00, 11:00, 13:00, 15:00 and 17:00 h) and on 5 consecutive days (at 09:00 h), respectively. MN reference intervals were based on the analysis of 120 plasma samples derived from healthy individuals in a sitting position (63 men, 57 women, age range 36 to 81 years, median age 54.5), during the PREVEND study[20,21]. Both studies were approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent. We calculated reference intervals by use of EP Evaluator[19].

We analyzed plasma samples from 10 patients with histologically proven pheochromocytoma to illustrate diagnostic value of the method.

Results

QUALITY CONTROL AND METHOD VALIDATION

Chromatography and selectivity

Total sample analysis time, including extraction, was 8 min. Complete chromatographic separation of the MNs is not necessary, whereas applying hydrophilic interaction chromatography (HILIC) chemistry reduces peak width and chromatographic time. Deuterated internal standards can be used, because the mass spectrometer monitors parent as well as daughter ions with high analytical specificity. Chromatograms obtained by XLC-MS/MS in MRM are shown in Fig. 2. We confirmed the identities of the compounds by the specific mass spectra. The mass chromatograms in Fig. 2, A–C, show the responses for MN, NMN, and 3-MT and the respective deuterated internal standards in a healthy individual, and Fig. 2, D–F, shows chromatograms with increased responses from a patient with histologically proven pheochromocytoma. Not all pheochromocytoma patients have increased plasma free 3-MT concentrations. Comparison of the chromatograms from the healthy individual with those of a pheochromocytoma patient reveals markedly increased MN (0.23 and 17.24 nmol/L, respectively; corresponding peak areas 2578 and 173771) and NMN (1.03 and 18.03 nmol/L; peak areas 1820 and 15253) concentrations in the patient

sample. In healthy individuals, both MN and NMN are present in low but quantifiable amounts.

Detection limits

LOD was 0.01 nmol/L for MN, 0.02 nmol/L for NMN, and 0.04 nmol/L for 3-MT. Respective quantification limits (at a signal-to-noise ratio of 10) were 0.03, 0.05, and 0.06 nmol/L, with CVs of 10%, 13%, and 16%.

Cartridges could be reused several times, with carryover <0.1% observed between sequential analyses performed on reused SPE cartridges, by applying additional washing steps in the method.

Linearity and imprecision

Plasma calibration curves and control samples were run with every batch of patient samples. Linearity was excellent over the 0 to 20 nmol/L calibration range, with corresponding correlation coefficients (R^2) consistently >0.99 for all 3 compounds. Plasma calibration curves were reproducible between days, with R^2>0.99. Mean analytical intra- and interassay repeatability and reproducibility for enriched pooled plasma in low, medium, and high concentrations are shown in Table 1. Intraassay CV (n=20) was 2.5% to 4.8% (MN), 5.1% to 6.2% (NMN), and 4.4% to 8.0% (3-MT). Interassay CV (n=20) was 3.4% to 5.6% (MN), 4.2% to 7.1% (NMN), and 4.5% to 11.1% (3-MT). Reproducibility, recovery, and imprecision were measured in the same way with aqueous calibration curves, which gave comparable results (data not shown). Plasma samples with high MN concentrations that exceed the calibration range can be diluted up to 100 times.

Recovery

Recoveries ranged from 94.4% to 99.6% (MN), 74.5% to 99.1% (NMN), and 81.4% to 98.5% (3-MT) and are shown in Table 2 for low, medium, and high concentrations.

Method comparison

We compared the results obtained by XLC-MS/MS and HPLC-ECD methods for patient specimens routinely screened for pheochromocytoma. Passing–Bablok regression for HPLC-ECD and XLC-MS/MS results (n=50) gave slopes of 1.19 (95% CI 1.02–1.62; R^2 0.9338) and 1.14 (1.06–1.25; 0.9675) and y-intercepts of 0.15 and 0.03 nmol/L for MN and NMN, respectively. No outliers were detected. 3-MT data could not be compared because with the HPLC-ECD method it is not possible to measure this marker in plasma.

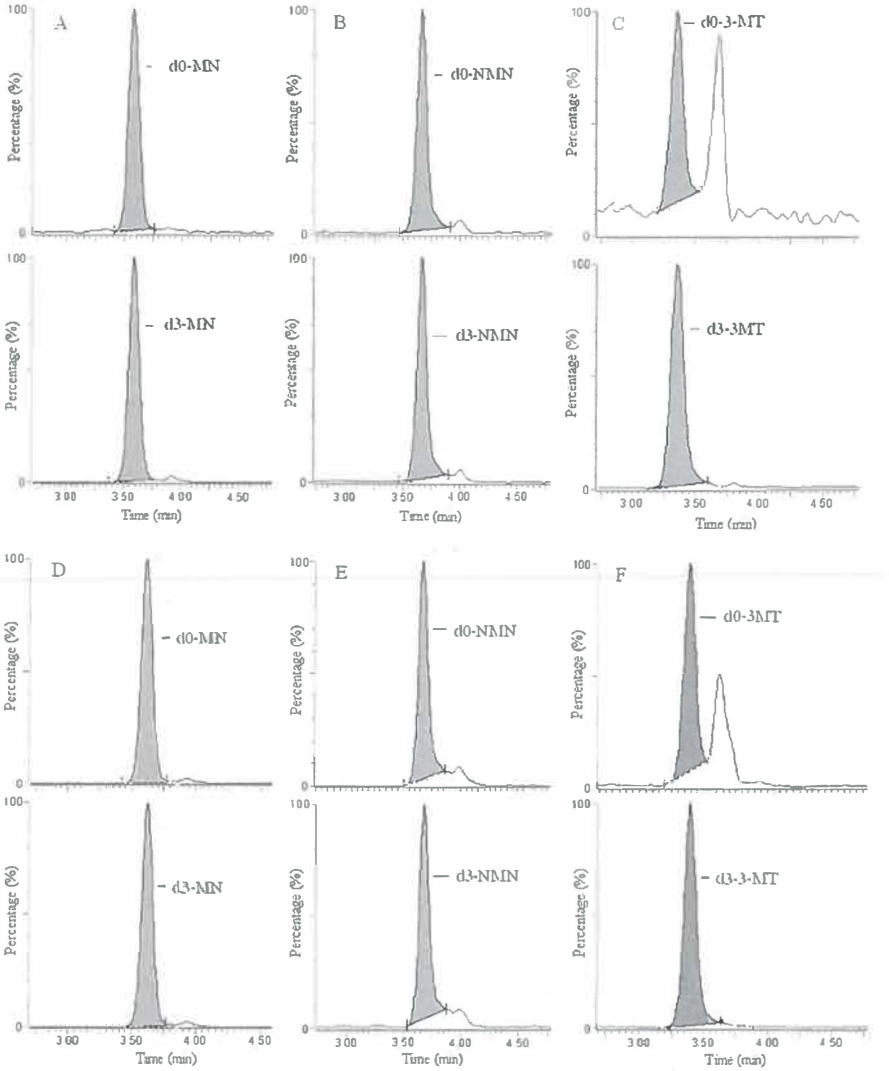


Figure 2. Chromatograms of free plasma MN, NMN, and 3-MT (d_0) and their deuterated internal standards (d_3) obtained by XLC-MS/MS in MRM mode.
A–C: MN (0.23 nmol/L; peak area d_0: 2578, d_3: 34499), NMN (1.03 nmol/L; d_0: 1820, d_3: 5544), and 3-MT (0.09 nmol/L; d_0: 566, d_3: 8754) in a healthy individual, respectively. *D–F:* MN (17.24 nmol/L; peak area d_0: 17377, d_3: 33151), NMN (18.03 nmol/L; d_0: 15253, d_3:2740), and 3-MT (1.07 nmol/L; d_0: 8223, d_3: 10298) in a patient with histologically proven pheochromocytoma. All signals are normalized to full scale for the highest peak in the window. Retention time is indicated in min. Parent–daughter transitions used for quantification were *m/z* 180→148 for MN, *m/z* 166→134 for NMN, and *m/z* 151→119 for 3-MT.
Abbreviation: *d*: deuterated.

Table 1. Intra- and interassay imprecision of the XLC-MS/MS method for plasma free MN, NMN, and 3-MT.

	Mean analytical variation (*n*=20)						**Mean biological variation (*n*=20)**					
	Intrassay			Interassay			Intraday			Interday		
	Mean, nmol/L	**SD,** nmol/L	**CV, %**	**Mean,** nmol/L	**SD,** nmol/L	**CV, %**	**Mean,** nmol/L	**SD,** nmol/L	**CV, %**	**Mean,** nmol/L	**SD,** nmol/L	**CV, %**
MN												
Low	0.53	0.01	2.50	0.23	0.01	5.55	0.22	0.02	9.43	0.26	0.02	8.41
Medium	1.66	0.08	4.82	1.41	0.08	5.41						
High	7.76	0.25	3.38	7.76	0.26	3.41						
NMN												
Low	0.59	0.03	5.37	0.59	0.04	7.08	0.47	0.07	15.18	0.50	0.07	13.44
Medium	1.65	0.10	6.18	1.68	0.11	6.34						
High	24.37	1.23	5.06	24.29	1.01	4.16						
3-MT												
Low	0.18	0.01	7.25	0.10	0.01	11.11	0.07	0.03	44.95	0.04	0.01	23.24
Medium	0.79	0.06	8.00	0.74	0.05	6.65						
High	3.03	0.13	4.42	3.04	0.13	4.25						

Analytical variation was calculated by measuring each sample 20 times per day (intraassay) and in 20 different assays (interassay).
Intra- and interday mean biological variation was calculated from results from healthy individuals at 5 times during a day (09:00, 11:00, 13:00, 15:00, and 17:00 h) and on 5 consecutive days at 09:00 h.

Table 2. Mean recoveries of the XLC-MS/MS method for plasma free MN, NMN, and 3-MT.

Compound	Recovery analyte, % (range)	Recovery IS, % (range)
MN		
Low	98.9 (97.3–99.6)	99.0 (98.3–99.4)
Medium	97.2 (94.4–99.5)	97.2 (95.9–98.8)
High	97.3 (95.8–98.6)	97.3 (96.1–98.6)
NMN		
Low	94.6 (90.7–99.1)	96.1 (93.7–98.3)
Medium	83.5 (76.7–95.2)	83.1 (77.8–94.6)
High	81.6 (74.5–93.7)	82.0 (75.0–89.8)
3-MT		
Low	91.1 (85.2–96.0)	96.6 (91.3–99.0)
Medium	90.4 (81.4–96.6)	94.6 (92.2–98.4)
High	96.6 (92.8–98.5)	96.7 (89.9–98.0)

Stability

MNs were stable in plasma stored up to 7 days at 10 or 4 °C. At room temperature, plasma MNs were stable up to 24 h. No changes in measured concentrations were observed in plasma that had been subjected to 1, 2, or 3 freeze-thaw cycles. Stability data (n=3) are not shown.

Biological variation, reference values, and patient samples.

Biological intra- and interday CVs (n = 10) were 9.4% and 8.4% (MN), 15% and 13% (NMN), and 45% and 23% (3-MT), as shown in Table 1.

The distribution of 120 reference values is right-shifted for all 3 MNs, as shown in Fig. 3. Therefore, we calculated reference intervals with EP evaluator in a transformed parametric manner according to CLSI C28-A2[22]. Reference intervals were 0.07 to 0.33 nmol/L (MN), 0.23 to 1.07 nmol/L (NMN), and <0.17 nmol/L (3-MT). Plasma free MN concentrations of 10 patients with histologically proven pheochromocytoma, measured with XLC-MS/MS, are shown in Table 3.

Table 3. Plasma free metanephrine concentrations (nmol/L) in 10 patients with histologically proven pheochromocytoma.

Patient	MN	NMN	3-MT
1	**17.24**	**18.03**	**0.76**
2	**17.93**	**11.92**	0.10
3	**2.04**	**6.78**	0.05
4	0.14	**4.05**	0.03
5	0.09	**56.80**	**2.63**
6	0.11	**70.10**	**2.62**
7	**14.62**	**5.41**	**64.89**
8	1.70	**12.59**	0.07
9	**3.58**	**13.24**	0.15
10	**15.18**	**59.44**	**0.47**

Plasma concentrations are given in nmol/L. Reference limits are 0.07–0.33 nmol/L for MN, 0.23–1.07 nmol/L for NMN, and <0.17 nmol/L for 3-MT.
Bold type values: Value exceeds upper reference limit.

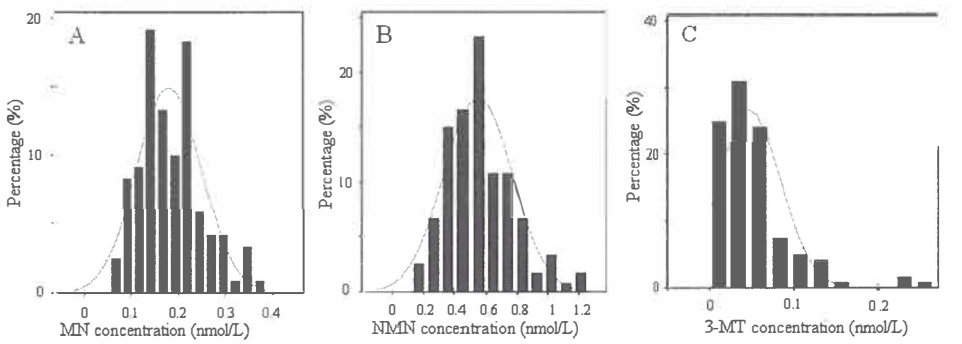


Figure 3. Distribution of fractionated plasma free MNs in reference samples from 120 healthy individuals in nmol/L.
A: MN; *B*: NMN; *C:* 3-MT.

Discussion

Measurement of *O*-methylated catecholamine metabolites (MNs) provides the best diagnostic accuracy for the biochemical diagnosis of pheochromocytoma[7,10,23]. Because most *O*-methylation takes place within the tumor cells that produce the catecholamines, the continuous production of MNs is independent of the highly variable sympathoadrenal catecholamine release in patients with pheochromocytoma. Consequently, concentrations of MNs are strongly correlated with tumor size[8]. Furthermore, diagnostic specificity of MN assays is increased with regard to catecholamine methods, as the concentrations of free MNs are less affected by environmental factors, including stress and changes of posture during specimen collection[1,2,4,8,10], although it was recently recommended that MN blood samples should be collected in a supine position[24,25,26]. Moreover, plasma MNs have higher stabilities than catecholamines, leading to simplified sample handling and storage[27,28].

Measurement of plasma free MNs is an analytical challenge, because these compounds occur in low concentrations without unique chemical characteristics and are difficult to isolate from the matrix. Several methods have been described for the determination of MNs, including HPLC with amperometric[13] or coulometric detection[29] and offline LC-MS/MS[16]. These methods have certain drawbacks such as labor intensity and long analysis times. Automated sample preparation reduces analysis time and analytical variation caused by differences in manual sample pretreatment. Furthermore, online concentration is possible by increasing the ratio between the sample and elution volume. Basic technique principles have been described previously for other applications[17,30]. Main advantages of XLC-MS/MS are ease of handling, portability, and reduction of cost per sample, because of reduced sample preparation time, high throughput, cheaper cartridges, and reuse of cartridges. Maintenance of the online extraction system is comparable to that of conventional HPLC systems, whereas maintenance costs are approximately triplicate.

Because MN, NMN, and 3-MT contain the same functional charged amino group, a selective SPE process can be achieved using cation exchange[12,29]. However, strong cation exchange media are not suitable for quaternary amines, because elution by neutralization is difficult. Such elution can be achieved when weak cation exchange material is used and the acidic mobile phase is applied to the cartridge in the chromatographic method. Oasis weak cation exchange cartridges (Waters) contain mixed-mode weak carbonyl cation-exchange material, which retains strong bases such as MNs, at pH >5, permitting the cartridge to be washed thoroughly with both water and 100% acetonitrile without elution of the analytes of interest.

The use of HILIC for the analysis of polar bases provides enhanced analytical sensitivity compared with traditional reversed-phase methods when using electrospray ionization. For

the desolvation process, an organic solvent is more efficient, and with HILIC, MNs are eluted in a high proportion of organic solvent (>80%). The principal is normal-phase separation in a reversed-phase manner with a polar stationary phase and an aqueous–organic mobile phase. This mode of chromatography is especially suitable for the separation of polar compounds from possible matrix interferences[31]. With MS/MS detection, high sensitivity is achieved, because unique parent-daughter ions are used for qualification and quantification. Interference with the same MRM transitions is eliminated chromatographically, whereas additional qualifiers enhance specificity of the detection method.

Correlation parameters show similarity between HPLC-ECD and XLC-MS/MS. However, XLC-MS/MS results in slightly higher concentrations than obtained with HPLC-ECD, which might be explained by the use of different calibration methods and internal standards. Reference intervals with XLC-MS/MS for MN and NMN (0.07–0.33 and 0.23–1.07 nmol/L, respectively) are in accordance with the ranges (0.05–0.47 and 0.12–1.1 nmol/L) measured by offline LC-MS/MS[16]. These limits are slightly lower for MN and higher for NMN than determined by the HPLC-ECD method (0.06–0.63 and 0.12–0.73 nmol/L)[13]. The difference in the upper reference limits for plasma free MN and NMN may be attributable to difference in internal standards (HMBA for HPLC-ECD; deuterated MNs for XLC-MS/MS) or sample collection position (sitting or supine).

The XLC-MS/MS method shows excellent linearity, and recoveries are consistent. The MNs are stable in plasma at 4 and 10 °C up to 7 days, which is in accordance with previous findings[27]. Extended storage is possible at –20 °C, because at least 3 freeze-thaw cycles had no influence on plasma MN concentrations.

The method allows reproducible quantification of plasma MNs. Analytical variation is <10% (except for low 3-MT concentrations), which is lower than in offline methods, owing to automation of the sample preparation[16,32]. Biological variation exceeds analytical variation for all 3 MNs.

Limits of quantification are decreased in comparison with non-MS methods[13] and detectable down to 0.05 nmol/L. In addition, the required plasma volume can be scaled down to 50 µL, which enables measurement of samples from infants and neonates. 3-MT concentrations can still be below the quantification limit in healthy individuals. In contrast, a few controls show increased concentrations of 3-MT (data not shown), which implies the necessity of further research before 3-MT concentrations can be used for the diagnosis of dopamine-dependent diseases such as extraadrenal paraganglioma[33].

Plasma samples from 10 histologically proven pheochromocytoma patients all showed NMN concentrations that considerably exceeded reference limits. MN and 3-MT concentrations were increased in some patients.

This automated quantification of plasma free MNs has been used successfully for 6 months in the routine biochemical analysis in our laboratory, in addition to the urinary analysis of MNs for the diagnosis of pheochromocytoma. XLC-MS/MS is a promising method that enables automated, high-throughput, accurate quantification of several other clinical important biomarkers.

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Chapter 4

Dietary Influences on Plasma and Urinary Metanephrines: Implications for Diagnosis of Catecholamine-Producing Tumors

Wilhelmina HA de Jong
Graeme Eisenhofer
Wendy J Post
Frits AJ Muskiet
Elisabeth GE de Vries
Ido P Kema

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Abstract

Measurements of the 3-O-methylated metabolites of catecholamines (metanephrines) in plasma or urine are recommended for diagnosis of pheochromocytoma. It is unclear whether these tests are susceptible to dietary influences. Aim was to determine the short-term influence of a catecholamine-rich diet on plasma and urinary fractionated metanephrines following a cross-over study in a specialist medical center involving 26 healthy adults. Subjects consumed catecholamine-rich nuts and fruits at fixed times on one day (about 35 μmol dopamine and 1 μmol norepinephrine) and catecholamine-poor products on another day. Blood and urine samples were collected at timed intervals before, during and after experimental and control interventions. Isotope-dilution mass spectrometry was applied to measure plasma and urinary concentrations of free and deconjugated 3-methoxytyramine (3-MT), normetanephrine (NMN) and metanephrine (MN). The catecholamine-rich diet resulted in substantial effects (up to 3-fold increases) on plasma concentrations and urinary outputs of free and deconjugated 3-MT. Dietary catecholamines had negligible influences on free NMN in plasma and urine, but substantial effects (up to 2-fold increases) on deconjugated NMN in plasma and urine. Concentrations of free and deconjugated MN in plasma and urine remained unaffected. In conclusion, dietary restrictions should be considered to minimize false-positive results for urinary and plasma deconjugated metanephrines during diagnosis of pheochromocytoma. Similar considerations appear warranted for plasma and urinary free 3-MT, but not for free NMN or MN, indicating advantages of measurements of the free compared to deconjugated metabolites.

Introduction

The diagnosis of pheochromocytoma depends on demonstration of elevated production of catecholamines, usually achieved by analysis of plasma and urinary free catecholamines and catecholamine metabolites. The 3-O-methylated metabolites (fractionated metanephrines), including metanephrine (MN) produced from epinephrine; normetanephrine (NMN) from norepinephrine; and 3-methoxytyramine (3-MT) from dopamine; are particularly useful[1-3]. The MNs are produced within tumor cells where the presence of membrane-bound catechol-O-methyltransferase leads to metabolism of catecholamines leaking from storage vesicles into the cytoplasm[4]. This process is continuous and independent of variations in catecholamine release, providing diagnostic advantages for MNs compared with the parent amines. Measurements of MNs in plasma or urine are therefore currently recommended for the diagnosis of pheochromocytoma[5-9], with some groups advocating use of the plasma test[5,6] and others the urinary test in combination with measurements of catecholamines[8,9].

We recently developed an automated online extraction-HPLC-tandem mass spectrometric method with high analytical performance for measurement of plasma free MNs[10]. However, biochemical results may be affected by preanalytical factors, such as physiological influences (*e.g.* exercise, posture, stress) and medications (*e.g.* catecholamine reuptake blockers) that alter the production or disposition of catecholamines and their metabolites. Dietary influences represent other factors that may affect biochemical tests of catecholamine excess. In particular, numerous food products, such as fruits and nuts, contain substantial quantities of biogenic amines that may produce false-positive test results[11-13].

Catecholamines and MNs are converted to sulfate conjugates (Fig. 1) by a sulfotransferase isoenzyme, SULT1A3, located in the gastrointestinal tract, that inactivates both endogenous and dietary-derived (exogenous) catecholamines[14-16]. Consequently, consumption of catecholamine-containing foods can lead to large increases in sulfate-conjugated catecholamines, particularly dopamine sulfate[15-17]. Because MNs in urine are commonly measured after an acid-hydrolysis deconjugation step and largely reflect sulfate-conjugated metabolites, an influence of food-derived catecholamines may be important to consider for those measurements. Although the influences of dietary catecholamines on biochemical test results have been studied previously[13,17-19], there are no clear data concerning these influences on concentrations of free or deconjugated MNs.

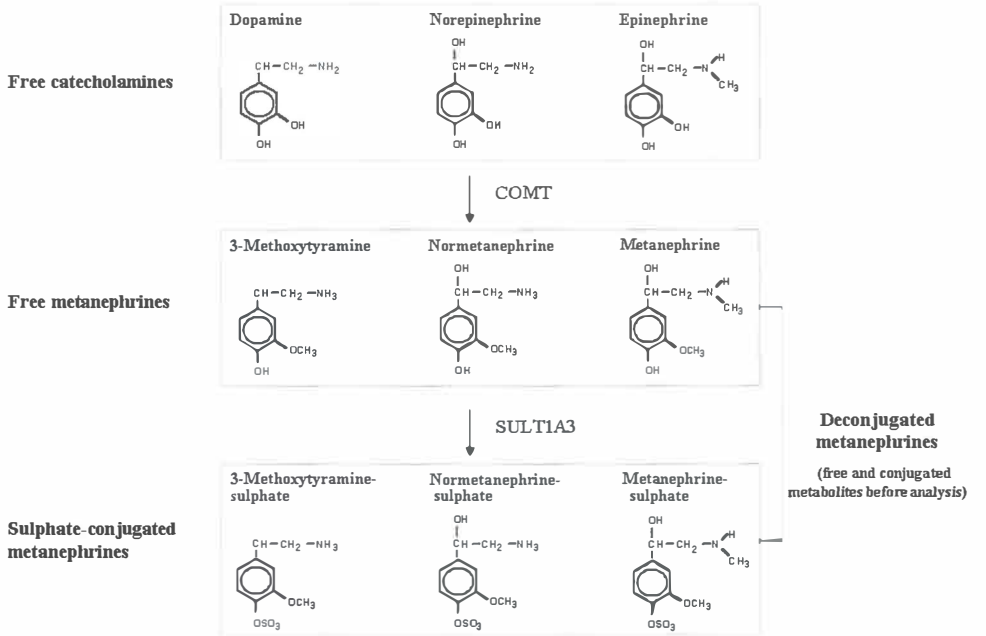

Figure 1. Simplified diagram showing the metabolism of catecholamines to free and sulfate-conjugated MNs before analysis. Dopamine, norepinephrine, and epinephrine are metabolized to free 3-MT, NMN, and MN, respectively, by the enzyme catechol-O-methyltransferase (COMT). The free MNs may then be conjugated with a sulfate group by the sulfotransferase isoenzyme 1A3 (SULT1A3). MNs may be measured in plasma or urine in either the free form or after a deconjugation step, the latter commonly employed for measurements of the urinary metabolites. The deconjugated MNs mainly represent sulfate-conjugated metabolites. This study included all four measurements (*i.e.* plasma free MNs, plasma deconjugated MNs, urinary free MNs, and urine deconjugated MNs).

The primary aim of this study was to determine the influence of a catecholamine-rich diet on free and deconjugated MNs. Plasma and urinary free and deconjugated MNs were measured to determine the relative influences of dietary constituents on each of the four tests. We also examined the influence of blood sampling in seated *vs.* supine positions to assess previous recommendations that samples should be collected after supine rest[6,20].

Materials and Methods

SUBJECTS AND DIET EXPERIMENT

Subjects included 26 healthy adults (13 women, 13 men; median age, 38 yr; range, 21–59 yr) who served as their own controls by participating in both control and experimental arms of the protocol following a crossover design, with at least 1 wk between the randomly distributed test days. Both arms were preceded by an overnight fast from at least 2400 h until 0830 h. Subjects avoided catecholamine-containing products (*e.g.* fruits, fruit drinks, nuts, potatoes, tomatoes, and beans) the day before, during, and the morning after both study days. The study design was based on a previous diet intervention study[13].

For the experimental arm, all subjects consumed two catecholamine-rich meals. The first meal, consumed at 0830 h, included two or three bananas (280 g of pulp), one fourth of a fresh pineapple (185 g of pulp), 50 g shelled walnuts, and 140 ml pineapple juice, purchased at local commercial outlets. At 1030 h each subject drank 280 ml pineapple juice. At 1230 h they consumed a second meal similar to the first. Finally, at 1430 h, participants drank 280 ml pineapple juice. Subjects avoided catecholamine-rich dietary products until the next morning. Based on previous measurements[13], total dopamine and norepinephrine intakes were estimated at 35 and 1 μmol, respectively; epinephrine was undetectable.

For the control arm, the subjects consumed meals (bread), snacks (gingerbread), and drinks (coffee, tea, dairy products) in accordance with the time schedule of the experimental arm. The study was approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants provided written informed consent.

SAMPLE COLLECTION

Morning urine until 0830 h was collected from all subjects. Four separate urine samples were then collected at 2-h intervals starting at 1030 h, with the fourth collection at 1630 h. A final urine specimen was collected beginning at 1630 h and ending at 0800 h the next morning (see Table 2). All urine samples were collected without acidification or preservative. Aliquots were stored within 1 d at –20 C until analysis within 6 months after collection.

Blood samples (10 ml) were collected into EDTA-containing Vacutainer tubes (Becton and Dickinson, Franklin Lakes, NJ) using an indwelling Braunule catheter (Braun, Melsungen, Germany) inserted into a forearm vein at 0800 h. The first blood sample was drawn immediately after insertion of the catheter with subjects in the seated position. A second blood sample was drawn after 30 min of supine rest (0830 h), immediately before the first meal. All subsequent blood samples were drawn after resting for 30 min in the supine

position. These samples were collected at 1030, 1230, and 1430 h before test meals or drinks, and finally at 1630 h. All blood samples were immediately centrifuged at 2,500 x *g* for 10 min at 4 C. Plasma samples were stored at –20 C until analyses within 3 months after collection. The procedures for urine and blood sampling were carried out identically on both control and experimental days.

Analytical methods

Plasma free and deconjugated MNs and urinary free MNs were measured using an online extraction-HPLC-tandem mass spectrometric method[10]. Plasma deconjugated MNs were determined after acid hydrolysis, carried out by incubating 1 ml of water-diluted (1:1) plasma with 15 µl of perchloric acid (pH 1.0) over 20 min at 100 C[21]. Urinary deconjugated MNs were determined by isotope-dilution gas chromatography-mass spectrometry, as previously described[22]. Urinary outputs of free and deconjugated MNs were normalized to the urinary excretion of creatinine, measured using an enzymatic method (Roche Diagnostics, Almere, The Netherlands), and expressed in units of micromoles per mole creatinine.

Reference intervals

Reference intervals for plasma deconjugated MNs were determined using blood samples collected in the seated position from 115 volunteers (57 males, 58 females; age range, 36–81 yr; median age, 55 yr) who participated in the PREVEND study[23]. Reference intervals for urinary free MNs were obtained from the analysis of 120 24-h urine samples that were collected from healthy subjects participating in the LifeLines study[24]. Reference values for plasma free and urinary deconjugated MNs have been reported elsewhere[10,22]. All reference intervals were determined without preceding dietary restrictions and calculated using EP Evaluator software (DG Rhoads Associates, Inc., Kennett Square, PA) as recommended by the Clinical and Laboratory Standards Institute.

Data analysis and statistics

Data are shown as mean values with 95% confidence intervals. The influence of blood sampling in seated *vs.* supine positions was tested using paired *t* test (SPSS version 16; SPSS Inc., Chicago, IL). The plasma samples collected at 0830 h and the first morning urine samples (until 0830 h) served as reference points for dietary-associated changes over time. Linear mixed models, tested for significance at $P < 0.05$, were used to determine the significance of temporal changes in analyte concentrations[25]. The model fit was evaluated on deviance and performed using the statistic software program ML Win version 2.0.2 (Centre for Multilevel Modeling, Bristol, UK). Changes over time were modeled in

agreement with the findings of descriptive statistics (SPSS). The magnitude of a difference between the control and the experimental groups in this modeling is given by the interaction term between diet and time because no differences at baseline concentrations between both groups were expected. Time was included as a covariate when possible, or otherwise as a factor.

Results

Plasma concentrations of free and deconjugated MNs exhibited divergent responses to dietary manipulations and the different postural conditions of blood sampling (Table 1).

Influence of sampling position

Plasma free NMN and MN were 30% and 12% higher, respectively ($P < 0.001$), in the blood sample collected in the seated position (08:00 h sample) than that collected in the supine position (08:30 h sample) after 30 min rest (Fig. 2). In contrast, there were no influences of these sampling conditions on plasma free 3-MT or deconjugated NMN, MN or 3-MT.

Influence of diet on plasma 3-MT

Plasma free 3-MT and deconjugated 3-MT differed significantly ($P < 0.05$) over the course of the day of the high-catecholamine diet compared with the control diet (Fig. 3, A&B). Plasma concentrations of both dopamine metabolites increased ($P < 0.05$) after the first catecholamine-rich meal, followed by stabilization until the next meal. After the second catecholamine-rich meal, plasma deconjugated 3-MT increased more than 3-fold compared with baseline levels ($P < 0.05$), whereas plasma free 3-MT increased 2-fold (Table 1 and Fig. 3, A&B). Both increases were followed by smaller but significant decreases. Plasma free 3-MT (Fig. 3A) showed no consistent change during the day of the control diet. In contrast, plasma deconjugated 3-MT (Fig. 3B) followed the same pattern observed on the day of the catecholamine-rich meals, albeit with much smaller but still significant increases after the consumption of each meal.

Table 1. Plasma concentrations of metanephrines before, during and after ingestion of catecholamine-rich and catecholamine-poor food products.

Time and position during blood sampling

Control	08.00 h Seated	08.30[a] h Supine	10.30 h Supine	12.30 h Supine	14.30 h Supine	16.30 h Supine	↑ Ref. limit
3-MT Free							0.17
Control	0.08 (0.08-0.09)	0.08 (0.07-0.08)	0.08 (0.07-0.09)	0.09 (0.08-0.09)	0.09 (0.08-0.09)	0.10 (0.08-0.11)	
Diet	0.08 (0.08-0.09)	0.09 (0.07-0.10)	0.15 (0.13-0.16)	0.15 (0.13-0.16)	0.18[b] (0.15-0.20)	0.14 (0.13-0.16)	
3-MT Deconjugated							19.12
Control	4.57 (4.00-5.14)	4.46 (3.93-4.99)	5.80 (5.15-6.46)	5.74 (5.12-6.37)	6.68 (5.87-7.48)	6.07 (5.36-6.78)	
Diet	5.44 (4.79-6.08)	5.35 (4.76-5.93)	12.08 (11.12-13.03)	13.28 (12.08-14.48)	18.49 (16.56-20.41)	16.76 (14.63-18.89)	
NMN Free							1.14
Control	0.54 (0.46-0.61)	0.41 (0.36-0.47)	0.43 (0.37-0.49)	0.47 (0.42-0.52)	0.43 (0.38-0.49)	0.46 (0.40-0.52)	
Diet	0.57 (0.50-0.63)	0.44 (0.38-0.49)	0.48 (0.41-0.56)	0.53 (0.46-0.59)	0.50 (0.43-0.57)	0.50 (0.44-0.57)	
NMN Deconjugated							39.03
Control	9.38 (7.96-10.79)	9.24 (7.95-10.53)	9.72 (8.33-11.12)	10.17 (8.87-11.48)	10.35 (9.04-11.67)	10.29 (9.01-11.57)	
Diet	10.19 (9.18-11.20)	10.13 (9.12-11.14)	15.00 (13.53-16.47)	14.22 (12.84-15.59)	18.35 (16.07-20.63)	16.50 (14.51-18.50)	
MN Free							0.34
Control	0.23 (0.20-0.26)	0.21 (0.18-0.24)	0.18 (0.16-0.21)	0.20 (0.18-0.23)	0.19 (0.16-0.22)	0.22 (0.18-0.25)	
Diet	0.24 (0.21-0.28)	0.21 (0.18-0.25)	0.20 (0.16-0.23)	0.21 (0.18-0.24)	0.20 (0.17-0.23)	0.21 (0.17-0.24)	
MN Deconjugated							12.26
Control	4.60 (3.78-5.41)	4.54 (3.80-5.29)	4.77 (3.93-5.61)	4.73 (3.96-5.49)	4.76 (4.04-5.47)	4.69 (4.00-5.39)	
Diet	5.05 (4.26-5.83)	5.09 (4.29-5.88)	5.47 (4.65-6.30)	5.31 (4.50-6.11)	5.52 (4.63-6.42)	5.42 (4.54-6.31)	

Test meals were taken at 08.30 h and 12.30 h; test drinks at 10.30 and 14.30 h. Mean plasma concentrations are given in nmol/L (n=26).
Data within parenthesis denote 95% confidence intervals. Deconjugated fractions refer to free + conjugated metanephrines.
Because of the used model fit statistics, significant changes over time are not expressed in this table.
Note: Upper limits of reference intervals (Ref. Limits) were determined from samples collected in the seated position and without dietary restrictions.
[a]: baseline time; [b]: above reference value.
Abbreviations: Ref. limit: reference limit, MN: metanephrine, NMN: normetanephrine, 3-MT: 3-methoxytyramine.

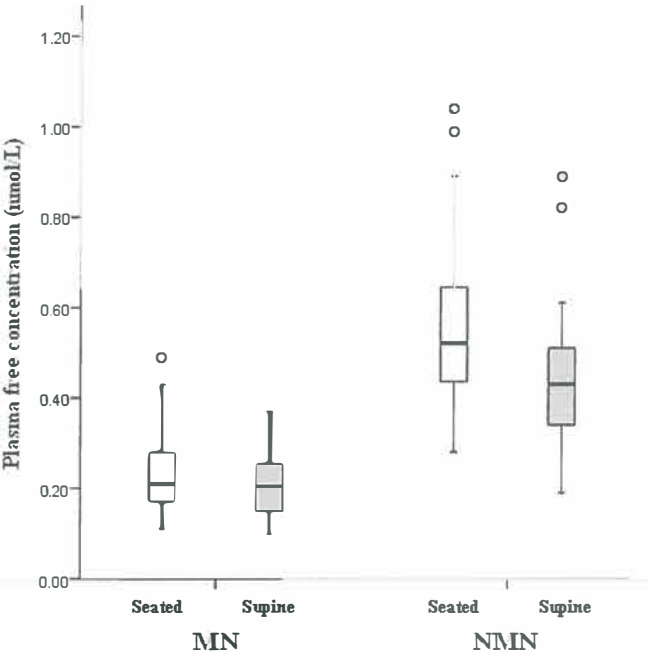

Figure 2. Differences in plasma concentrations of free MN and NMN after blood sampling in the seated position compared with after 30 min of supine rest. Data from 26 subjects are shown graphically. Decreases in mean MN and NMN concentration after 30 min of supine rest are significant ($P < 0.001$). The *single dots* are outliers.

INFLUENCE OF DIET ON PLASMA NMN

In contrast to the results for plasma 3-MT (Fig. 3A), plasma free NMN showed no significant differences during the days that subjects consumed the catecholamine-rich meals compared with the control meals (Fig. 3C). Concentrations in both groups gradually increased during the day, starting after the first supine baseline sample at 0830 h. In contrast to free NMN (Fig. 3C), but similar to plasma deconjugated 3-MT (Fig. 3B), plasma deconjugated NMN differed significantly ($P < 0.05$) after high-catecholamine meals compared with control meals (Fig. 3D and Table 1). More specifically, plasma deconjugated NMN showed successive increases ($P < 0.05$) after each of the two meals, with smaller but significant intervening decreases thereafter. After the second meal, levels of deconjugated NMN increased nearly 2-fold compared with baseline values. Plasma deconjugated NMN also increased slightly after control meals, but the increase only reached significance after the second meal.

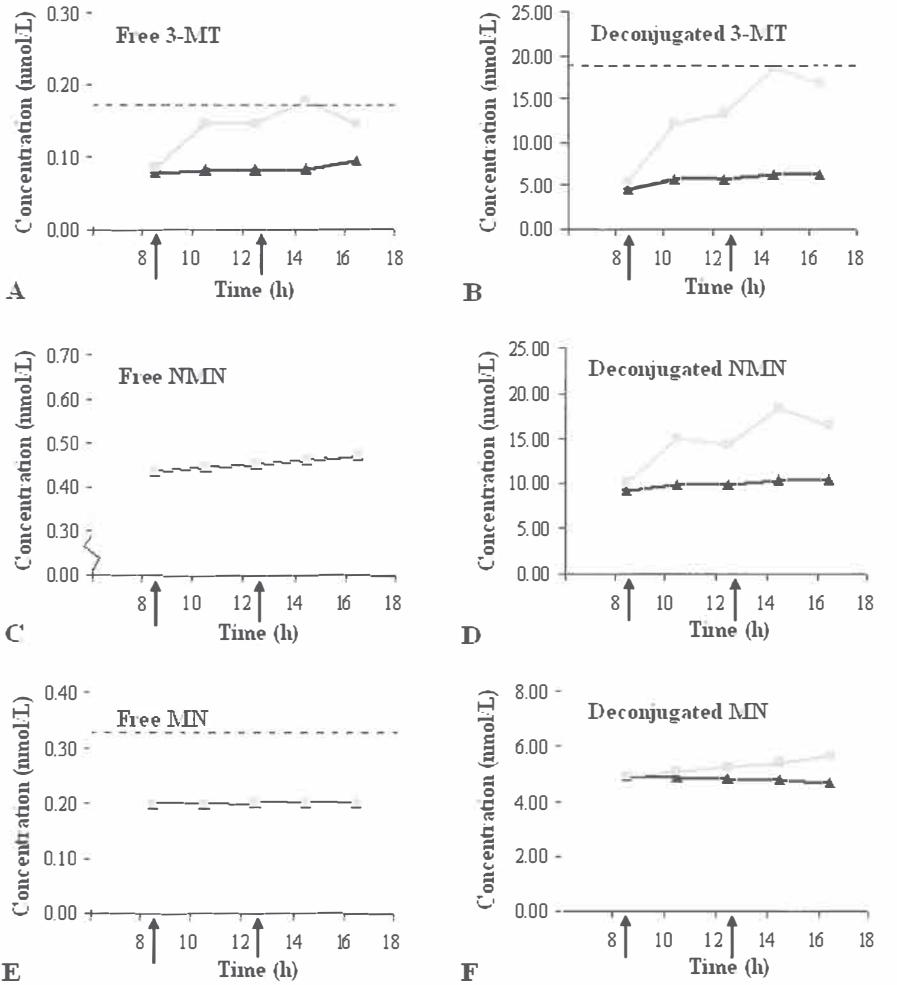

Figure 3. Line graphs of plasma concentrations of metanephrines before, during and after catecholamine-rich and catecholamine-poor meals obtained from statistical linear mixed model fit. Models were calculated from data of 26 subjects in both the high catecholamine diet and control arms of the study. Changes in concentrations in these models are significant ($P < 0.05$). Control and diet group data are significantly different ($P < 0.05$) from each other when lines do not coincide. The obtained models reflect a true illustration of the raw data (mean ±95% confidence intervals) (Table 1) after normalization for differences in baseline values (which for the model fit were assumed not to differ on experimental and control arms of the study). *A*: plasma free 3-MT. *B*: plasma deconjugated 3-MT. *C*: plasma free NMN. *D*: plasma deconjugated NMN. *E*: plasma free MN. *F*: plasma deconjugated MN. *Arrows* indicate the times at which test meals were taken (8.30 h a.m. and 12.30 h). *Grey line*: model for the diet group (n=26); *black line*: model for the control group (n=26). Models for plasma free NMN (C) and free MN (E) in diet and control group are calculated to be similar, therefore both lines coincide. The *dotted line* indicates the upper reference limit of the analyte in the graph. Abbreviations: MN: metanephrine, NMN: normetanephrine, 3-MT: 3-methoxytyramine.

Table 2. Urinary concentrations of metanephrines before, during and after ingestion of catecholamine-rich and catecholamine-poor food products.

	Time						
Control	**Morning urine-08.30[a] h**	**8.30-10.30 h**	**10.30-12.30 h**	**12.30-14.30 h**	**14.30-16.30 h**	**16.30-08.30 h**	**↑ Ref. limit**
3-MT Free							46
Control	23.9 (21.3-26.5)	23.8 (21.2-26.3)	26.4 (23.1-29.9)	25.8 (22.3-29.4)	26.7 (23.1-30.3)	22.8 (19.9-25.7)	
Diet	23.1 (20.8-25.4)	28.8 (25.2-32.3)	36.0 (32.1-39.9)	40.3 (34.9-45.7)	37.0 (32.5-41.5)	25.6 (22.9-28.3)	
3-MT Deconjugated							197
Control	80 (66-95)	67 (57-78)	81 (67-95)	85 (70-100)	87 (73-101)	80 (68-91)	
Diet	78 (62-95)	81 (67-95)	124 (108-140)	154 (132-176)	165 (146-183)	104 (91-118)	
NMN Free							29
Control	11.5 (10.0-13.1)	16.2 (14.3-18.1)	18.3 (15.9-20.6)	17.6 (15.5-19.7)	18.4 (15.6-21.1)	13.1 (11.4-14.7)	
Diet	10.8 (9.2-12.4)	16.8 (15.0-18.6)	19.9 (18.0-21.9)	20.4 (18.4-22.5)	20.4 (18.1-22.8)	16.1 (14.2-18.0)	
NMN Deconjugated							260
Control	118 (104-132)	113 (97-129)	124 (107-141)	130 (113-146)	135 (116-153)	125 (109-140)	
Diet	115 (103-128)	122 (109-134)	193 (170-216)	248 (220-276)	304[b] (269-339)	239 (206-272)	
MN Free							20
Control	11.3 (9.7-13.0)	11.9 (10.3-13.4)	13.2 (11.5-14.9)	12.6 (10.9-14.3)	13.6 (11.7-15.6)	11.1 (9.5-12.6)	
Diet	11.4 (9.8-13.0)	12.6 (10.9-14.3)	12.0 (10.5-13.6)	11.9 (10.3-13.5)	12.0 (10.3-13.7)	11.9 (10.2-13.6)	
MN Deconjugated							99
Control	57 (49-66)	56 (48-64)	63 (53-73)	64 (56-73)	65 (56-74)	58 (50-65)	
Diet	58 (51-66)	60 (52-68)	62 (55-70)	65 (56-74)	65 (57-73)	59 (50-68)	

Test meals containing catecholamines were taken at 08.30 h and 12.30 h; drinks at 10.30 and 14.30 h. Urinary concentrations are given in µmol/mol creatinine (n=26). Data within parenthesis denote 95% confidence intervals. Deconjugated fractions refer to free + conjugated metanephrines. The first urine collection consisted of first morning urine and all urine excreted after that until 08.30 h.
Note: Upper limits of reference intervals were obtained without dietary restrictions, indicating undervalued upper limits. In addition, limits may be distorted because they were determined in 24h urine collections instead of 2h and overnight portions.
Because of the used model fit statistics, significant changes over time are not expressed in this table.
[a]: baseline time; [b]: above reference value.
Abbreviations: Ref. limit: reference limit, MN: metanephrine, NMN: normetanephrine, 3-MT: 3-methoxytyramine.

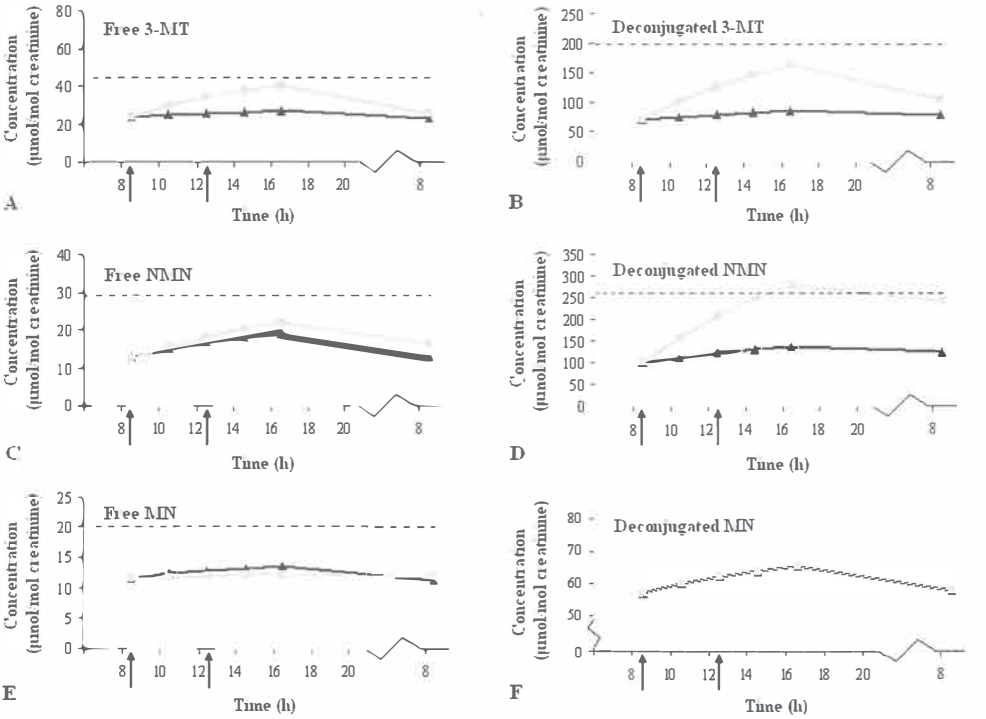

Figure 4. Line graphs of urinary concentrations of metanephrines before, during and after catecholamine-rich and catecholamine-poor meals obtained from statistical model fit. Models were calculated from data of 26 subjects in both the control and the diet group. Changes in concentrations in these models are significant ($P < 0.05$). Control and diet group data are significantly different ($P < 0.05$) from each other when lines do not coincide. The obtained models reflect a true illustration of the raw data (mean ±95% confidence intervals) (Table 2) after normalization for differences in baseline values (which for the model fit were assumed not to differ on experimental and control arms of the study). *A*: urinary free 3-MT. *B*: urinary deconjugated 3-MT. *C*: urinary free NMN. *D*: urinary deconjugated NMN. *E*: urinary free MN. *F*: urinary deconjugated MN.
Arrows indicate the times at which test meals were taken (08.30 and 12.30 h). *Grey line*: significant model for the diet group (n=26); *black line*: significant model for the control group (n=26). Models for urinary deconjugated MN (F) in diet and control group are calculated to be similar, therefore both lines coincide.
Abbreviations: MN: metanephrine, NMN: normetanephrine, 3-MT: 3-methoxytyramine.

Influence of diet on plasma MN

Similar to plasma free NMN (Fig. 3C), plasma free MN did not differ between the days that subjects ingested catecholamine-rich and control meals (Fig. 3E). Levels remained constant over the course of both days. For plasma deconjugated MN (Table 1 and Fig. 3F), there were large interindividual variations that made detection of between-group differences difficult. However, following the model fit formula, the significant increase ($P < 0.05$) of plasma deconjugated MN in the diet group appeared similar to that of the control group (Table 1), which implies no influence of the catecholamine-rich diet on plasma deconjugated MN.

Influence of diet on urinary 3-MT

Urinary outputs of free and deconjugated 3-MT were significantly ($P < 0.05$) higher after ingestion of the high-catecholamine meals compared with control meals. After consumption of the second catecholamine-rich meal, urinary outputs of free 3-MT (Table 2 and Fig. 4A) were nearly 2-fold ($P < 0.05$) and deconjugated 3-MT more than 2-fold higher ($P < 0.05$) than baseline values (Table 2 and Fig. 4B). Urinary free 3-MT returned to baseline values overnight, whereas urinary deconjugated 3-MT remained elevated, but decreased significantly from values of the preceding collection. No significant changes in urinary concentrations of free or deconjugated 3-MT were apparent after ingestion of the control meal.

Influence of diet on urinary NMN

Urinary free NMN showed similar daytime increases and nighttime decreases ($P < 0.05$) during the 24-h period after both the catecholamine-rich and control meals (Fig. 4C). The observed small difference in the model fit after control and catecholamine-rich meals was not significant, indicating no influence of the high-catecholamine diet on urinary free NMN. In contrast to urinary free NMN (Fig. 4C), urinary deconjugated NMN showed significant ($P < 0.05$) differences after ingestion of control *vs.* experimental meals up until the following morning (Fig. 4D). On the day of the catecholamine-rich diet, urinary deconjugated NMN increased by nearly 3-fold over baseline values for the collection between 1430 and 1630 h and remained 2-fold higher for the overnight collection.

Influence of diet on urinary MN

Urinary free (Fig. 4E) and deconjugated MN (Fig. 4F) showed the same time course of changes after both control and experimental meals, indicating no influence of the

catecholamine-rich diet. Urinary deconjugated MN, and to a lesser extent urinary free MN, both showed significant ($P < 0.05$) increases during the day, followed by decreases at night.

Discussion

This study shows that dietary catecholamines can dramatically influence plasma as well as urinary concentrations of the 3-O-methylated metabolites of catecholamines. More specifically consumption of catecholamine-rich food products results in sustained and substantial increases in plasma and urinary deconjugated NMN and 3-MT, smaller increases in free 3-MT, but negligible effects on free NMN and MN. These data are consistent with other findings showing that the gastrointestinal tract provides a major site for sulfate conjugation of catecholamines[15,16], that diet can profoundly influence levels of sulfate conjugates[14,16-18,26-28], and that the free MNs have a more rapid circulatory clearance than conjugated metabolites with different sites of production[29]. The present study extends these earlier findings by providing novel data about dietary influences on free and deconjugated MNs in urine and plasma, with important implications for biochemical testing of catecholamine-producing tumors.

Our study further shows that whereas the free MNs are relatively insensitive to dietary factors, they are quite sensitive to the conditions of blood sampling, showing rapid decreases within 30 min of supine rest after insertion of an iv catheter. The drops in plasma free NMN and MN after supine rest reflect decreased release of norepinephrine and epinephrine that is consistent with previous findings of posture-associated changes in plasma MNs[20,30]. The lesser effect on MN than NMN is attributable to the substantial amount of circulating MN produced within adrenal medullary cells independently of epinephrine release[4]. Lack of influence of supine rest on plasma deconjugated MNs is explained by the much slower circulatory clearance of the sulfate conjugate than the free metabolites[29,31,32]. The daytime increases and nighttime decreases in circulatory outputs of urinary free and deconjugated NMN and MN are consistent with previous observations[19,33.34] that likely reflect increased sympathoadrenal outflow related to a more ambulatory and active status during waking hours.

Lack of effect of the high-catecholamine diet on free NMN in plasma and urine, but the substantial effects on deconjugated NMN, reflect the importance of sulfate conjugation for metabolism of dietary catecholamines[14-16] and the different sources of free and sulfate-conjugated NMN[29]. The increases in both free and deconjugated 3-MT after the high-catecholamine diet are also consistent with previous observations of the more sensitive nature of free dopamine and its metabolites to dietary catecholamines[16,35]. The higher amounts of dopamine than of norepinephrine and negligible amounts of epinephrine in food

products presumably also contribute to the above differences and lack of influence of diet on plasma and urinary MN.

Catecholamines have a relatively low abundance in food products compared with serotonin. Consequently, dietary restrictions have generally been considered unnecessary for diagnosis of catecholamine-producing pheochromocytomas[13]. In recent years, however, there has been a shift in the way these tumors are diagnosed. Rather than focusing on measurements of plasma or urinary free catecholamines, which are relatively insensitive to dietary influences, the focus today is on measurements of plasma or urinary MNs, now recognized to offer superior diagnostic sensitivity compared with the parent amines[1-3,5-7]. Although there have been a few studies indicating an influence of diet on urinary deconjugated MNs[36,37], others have indicated little influence[19,38], and until now it has remained unclear whether the conjugated or free metabolites are susceptible to any effect of diet.

The present study is, to our knowledge, the first to investigate the short-term influence of a catecholamine-rich diet on free and deconjugated MNs in plasma and urine using modern analytical techniques characterized by a combination of high sensitivity, specificity, and precision[10]. Although the total amounts of specific catecholamine-rich foods (*e.g.* six bananas) ingested may seem unusual from the standpoint of a typical Western diet, it should be appreciated that there are many other food products besides fruits and nuts capable of similar influences (*e.g.* tomatoes, beans, and other vegetables; cheeses; fermented foods; processed meat products). Most are likely unrecognized. In one study involving ingestion of a single ordinary meal, for which there was no consideration of catecholamine content, plasma levels of dopamine sulfate increased by 46-fold with lesser increases in the sulfate conjugates of norepinephrine and epinephrine[16]. Similarly large increases in plasma dopamine sulfate were also observed in another study involving ingestion of ordinary meals[26]. In that study, contents of catecholamines in one of the three meals that were consumed were more than 10-fold higher than the total amounts of catecholamines consumed in all the meals combined in the present study. Thus, by this standard of an ordinary meal, the total amounts of catecholamines consumed in the present study cannot be considered unusual.

Dietary influences may not, however, be confined to food products that contain catecholamines. Cereals and wheat germ-containing products, which contain negligible amounts of catecholamines, are also capable of eliciting large increases in dopamine sulfate. The mechanism appears to involve the presence of tyrosinase, which converts tyrosine to L-dopa[27]. Additionally, tyramine in the diet may be metabolized by mixed-function oxidases and other enzymes to dopamine and 3-MT[39].

With the above in mind, the results of the present study indicate a need to reconsider dietary restrictions during the laboratory diagnosis of catecholamine-producing tumors. Although

dietary restrictions appear unnecessary for measurements of free NMN and MN in plasma or urine, our data indicate that such restrictions may be important for measurements of deconjugated NMN, MN, 3-MT, and also free 3-MT in both matrices. The simplest countermeasure to minimize any dietary influence on free 3-MT is an overnight fast, a precaution recommended previously for measurements of plasma free MNs[30]. Whether an overnight fast is sufficient for plasma deconjugated MNs is unclear. As illustrated by the incomplete fall in morning urinary deconjugated MNs after the high-catecholamine diet, fasting does not appear sufficient for overnight urine samples and is impractical for 24-h collections. For such collections, avoidance of catecholamine-rich products seems appropriate.

Among three studies of diagnostic tests for pheochromocytoma that included measurements of plasma free and urinary deconjugated NMN and MN[2,5,40], all indicated markedly better diagnostic performance of both tests compared with the parent catecholamines and moderately better performance of plasma free than urinary deconjugated NMN and MN. Associated findings of up to 3-fold more false-positive results for urinary deconjugated than free NMN and MN[5] may be related to dietary influences on conjugated but not on free metabolites. This possibility may be explored in future studies employing dietary restrictions before testing for the tumor. Patients with positive urinary deconjugated MN results could be alternatively retested after avoidance of catecholamine-rich food products.

The above suggestion is in line with similar recommendations for plasma free MNs, where findings of positive test results after blood sampling in the seated position should be followed by testing in the supine position[6,20]. Our data support that and the related recommendation that reference intervals for plasma free MNs should be established in samples taken after supine rest[20]. By similar reasoning, it also seems appropriate to recommend that reference intervals for urinary and plasma deconjugated MNs should be established with consideration of dietary influences.

The reference values for plasma free MNs outlined in the present study were established by blood sampling in the seated rather than the recommended supine position and need to be reestablished. The subsequent lower upper limits would, however, also be expected to result in an increased likelihood of false-positive test results should the appropriate corresponding precautions not be taken during diagnostic testing. However, the relatively easy precautions of an overnight fast plus blood samples collected in the supine position for measurement of free MNs compared with longer-term dietary restrictions required for measurement of deconjugated MNs indicate further advantages for the determination of free over deconjugated metabolites.

Conclusions and recommendations

Catecholamine-rich food consumption has no clinically relevant effect on concentrations of plasma and urinary free NMN, free MN, and deconjugated MN. Therefore these analytes can be determined without preceding dietary restrictions. In contrast, dietary restrictions are indicated for measurements of plasma and urinary free 3-MT, deconjugated 3-MT, and deconjugated NMN. The diet dependency of urinary deconjugated NMN is potentially important because measurements of urinary deconjugated MNs are commonly used for the diagnosis of pheochromocytoma. To improve diagnostic performance, consideration should be given either to employing dietary restrictions or to alternative measurements of urinary or plasma free MNs. Our data also imply that dietary restrictions are necessary for the measurements of 3-MT as a biochemical marker for dopamine-producing pheochromocytomas and neuroblastomas.

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Chapter 5

Automated mass spectrometric analysis of Urinary Free Catecholamines Using On-Line Solid Phase Extraction

Wilhelmina H A de Jong
Elisabeth GE de Vries
Bruce HR Wolffenbuttel
Ido P Kema

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Abstract

Background: Analysis of catecholamines (epinephrine, norepinephrine and dopamine) in plasma and urine is used for diagnosis and treatment of catecholamine-producing pheochromocytoma. Current analytical techniques for catecholamine quantification are laborious, time consuming and technically demanding. Aim was to develop an automated on-line solid-phase extraction method coupled to high performance liquid chromatography-tandem mass spectrometry (XLC-MS/MS) for the quantification of free catecholamines in urine.

Methods: Five µL urine equivalent was pre-purified by automated on-line solid-phase extraction, using phenylboronic acid (PBA) complexation. Reversed phase (pentafluorophenylpropyl column) chromatography was applied. Mass spectrometric detection was operated in multiple reaction monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionisation. Urinary reference intervals were set in 24h-urine collections of 120 healthy subjects. XLC-MS/MS was compared with liquid-chromatography with electrochemical detection (HPLC-ECD).

Results: Total run-time was 14 min. Intra- and inter-assay analytical variation were <10%. Linearity was excellent (R2>0.99). Quantification limits were 1.47 nmol/L, 15.8 nmol/L and 11.7 nmol/L for epinephrine, norepinephrine and dopamine, respectively. XLC-MS/MS correlated well with HPLC-ECD (correlation coefficient >0.98). Reference intervals were 1-10, 10-50 and 60-225 µmol/mol creatinine for epinephrine, norepinephrine and dopamine, respectively.

Conclusions: Advantages of the XLC-MS/MS catecholamine method include its high analytical performance by selective PBA affinity and high specificity and sensitivity by unique MS/MS fragmentation.

Introduction

Analysis of urinary catecholamines (i.e. epinephrine, norepinephrine and dopamine) is used for the diagnosis pheochromocytoma and paraganglioma which are catecholamine-producing tumors. Traditionally, biochemical diagnosis of pheochromocytoma is based on the presence of catecholamines and/or their metabolites in urine and plasma. Although the measurement of free metanephrines (3-O-methylated catecholamine-metabolites) in plasma or urine is recommended[1-5], urinary metanephrines in combination with measurements of catecholamines are commonly applied[4,5]. This means that there is still a place for measurements of urinary catecholamines in clinical chemistry. However, rather complex analytical methods are required for these compounds, since catecholamines are present at the nanomolar concentration range and are susceptible to oxidation. Therefore, sensitive and selective methods are required to determine their precise concentrations. Traditionally, high performance liquid chromatography (HPLC) is applied with electrochemical or fluorometric detection[6,7]. Usually, analysis times are long, mainly caused by extensive and technically demanding sample preparation with ion-pair or derivatisation reagents. In addition, these methods are susceptible to interferences caused by co-eluting compounds, which complicates the interpretation of results[8].

In the last decade liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) methods have been developed[9-11] for analyzing free catecholamines in urine with high sensitivity and specificity. Still, laborious sample preparation is required, usually performed with activated alumina, cation-exchange adsorbents or phenylboronic acid (PBA) complexation[12]. These principles do enable automated on-line analysis of urine samples[13]. Currently, on-line coupling of these catecholamine sample preparation principles directly to LC-MS/MS has not been shown. Our aim was to develop an automated on-line sample preparation LC-MS/MS method for the quantification of free catecholamines in urine in order to replace the current laborious and time-consuming conventional HPLC method.

Materials and Methods

Chemicals and Reagents

HPLC-grade methanol was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK); ammonium formate 99.995+%, ammonium hydroxide and acetic acid from Sigma-Aldrich Ltd. (Steinheim, Germany); formic acid 98% to 100% ultrapure from BDH Laboratory Supplies (Poole, UK); urine preservatives hydrochloric acid, ascorbic acid and EDTA were acquired from Merck KGaA (Darmstadt, Germany), as was ammonium chloride (p.a. quality).

Epinephrine, norepinephrine and dopamine-HCl were obtained from Sigma-Aldrich Ltd. Epinephrine-N-methyl-d3, norepinephrine-2,5,6,α,β,β-d6 and dopamine-1,1,2,2,-d4-HCl were from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada); Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure. All chemicals and solvents were of analytical reagent grade.

INSTRUMENTS

A Spark Holland Symbiosis® on-line solid phase extraction (SPE) system (Spark Holland, Emmen, the Netherlands) was used for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (2 high-pressure dispensers), and an HPLC pump, as shown previously[14-16]. The ACE module contains 2 connectable 6-way valves and a SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and cleanup. The integrated HPLC pump was a binary high-pressure gradient pump. Column temperature was controlled with a Mistral Column Oven (Spark Holland). Detection was performed with a Xevo® TQ tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters, Milford, MA). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software with automated data processing using the TargetLynx Application Manager (Waters).

SAMPLE PREPARATION

A catecholamine mix working solution was diluted with ascorbic acid (400 mg/L) from separate stock solutions (8.5-60 mg/mL) in methanol on the day of analysis. Stock solutions were stored at -20 °C for a maximum of 6 months. Deuterated internal standard stock and mix working solution were treated the same way. Urinary calibrators were prepared by addition of working solution mix in the concentration ranges 0-1400 nmol/L for epinephrine, 0-5000 nmol/L for norepinephrine and 0-9600 nmol/L for dopamine. All solutions were prepared in blank urine with ascorbic acid solution (400 mg/L) added as preservative. Low, medium and high quality-control samples were made by spiking and dilution of urine from a healthy control. Urine samples were stored at –20°C until analysis. Blank urine without the presence of catecholamines was obtained by oxidation of regular urine under alkaline conditions at 100 °C followed by acidification to pH 4. Fifty µL of urine (acidified with HCl to pH 4 and containing the preservatives ascorbic acid and EDTA (1:1 w/w), added prior to collection) was mixed directly in an autosampler vial with 100 µL internal standard solution (final concentration 12.3 nmol/L for epinephrine, 66.6 nmol/L for

norepinephrine and 90.5 nmol/L for dopamine). Urine samples were diluted with ascorbic acid solution (400 mg/L) to reach a final volume of 1 mL. 100 µL sample was injected into the XLC-MS/MS system. This injection volume was equivalent to 5 µL of urine.

On-line SPE

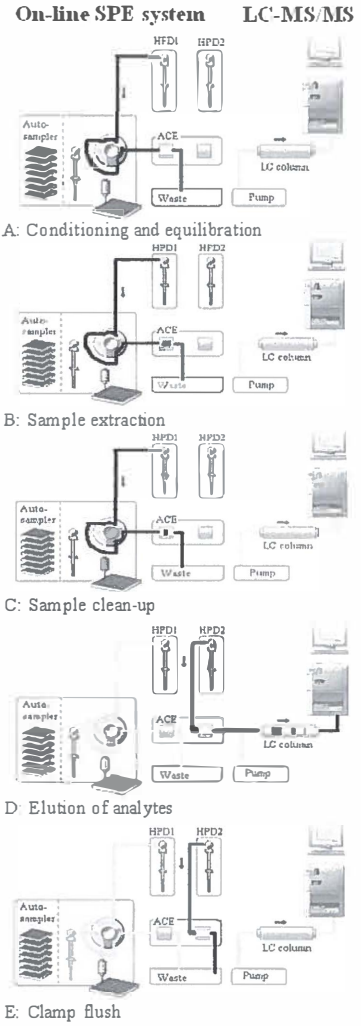
Sample clean-up took place by on-line SPE, following the technique as described previously[14-16]. Bond Elut® PBA (phenyl boronic acid; Varian Inc. Palo Alto, CA) 10 by 2 mm SPE cartridges (Spark Holland) were used for sample extraction. The Symbiosis® system was designed to proceed automatically through a series of programmable routines (Fig.1) during which the SPE cartridge is conditioned (Fig. 1A), loaded (with 200 mM ammonium chloride buffer pH 8, Fig. 1B), washed (with methanol and water, Fig. 1C), and eluted (250 µL 100 mM ammonium formate pH 3, Fig 1D). Solvents were delivered by two high pressure dispensers. The eluted fraction was directly mixed with the chromatographic mobile phase and transferred to the analytical column (Fig 1D). Processing of subsequent samples was carried out in parallel and cartridges and tubing were flushed and regenerated (Fig. 1E).

Liquid chromatography

Chromatographic separation was achieved by using an Allure PFP (pentafluorophenyl) propyl column (particle size 5 µm, 4.6 mm internal diameter by 150 mm; Restek, Bellevonte, PA). A gradient flow starting with 80% of 25 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid (A) and 20 % of methanol (B) and a flow rate of 0.50 mL/min was applied to the chromatographic column (Table 1). Gradients applied were linear. Column temperature was kept at 25 °C.

Mass spectrometry

The mass spectrometer was directly coupled to the chromatographic column. In positive electrospray ionization mode epinephrine, norepinephrine and dopamine were protonated to produce ions at the form $[M+H]^+$. Upon collision-induced dissociation (CID) with argon gas, these precursor ions produced characteristic product ions of [M+H-H2O] (Table 2). Other mass transitions monitored were used as qualifiers (Table 2), following EU directive 2002/657/EC.



A: Conditioning and equilibration
A1: New cartridge — From tray to left clamp
A2: Conditioning
A3: Equilibration

B: Sample extraction
B1: Injection — Start loopfill with sample
B2: Extraction — Sample loading using 200 mM ammonium chloride pH8

C: Sample clean-up
C1: Wash cartridge — Methanol/Water (90/10)
C2: Wash cartridge — Methanol/Water (10/90)

D: Elution of analytes
D1: Transfer cartridge — From left to right clamp
D2: Elution — Elution of analytes from cartridge To LC column using HPD2
D3: New cartridge — From tray to left clamp

E: Clamp flush
E1-E5: Cartridge wash and re-equilibration
E6: Move cartridge — From right clamp to tray

Figure 1. Schematic representation of the on-line solid phase extraction.
A: conditioning and activation of the SPE cartridge in the left clamp. B: sample extraction after filling of the autosampler loop. C: sample cleanup. D: right clamp elution of analytes, mixture with the chromatographic liquids, chromatographic separation and mass spectrometric detection. E: cartridge and clamp cleanup.
Abbreviations: SPE: solid phase extraction; HPD: high-pressure dispenser; LC: liquid chromatography; ACE: automatic cartridge exchanger, MS/MS: tandem mass spectrometry.

Table 1. Gradient elution scheme liquid chromatography

Time (mm:ss)	Flow (mL/min)	Solvent A (%)	Solvent B (%)
00:01	0.50	90	10
00:02*	0.10	90	10
00:36*	0.10	90	10
00:37	0.50	90	10
06:00	0.50	40	60
11:30	0.50	40	60
12:00	0.50	90	10
14:00	0.50	90	10

Solvent A: 25 mM ammonium formate pH 3; Solvent B: Acetonitrile
* Total column flow is 0.50 mL/min due to mixing with elution solvent.

URINE SAMPLES

For method-comparison studies, 59 urine samples from the routine catecholamine analysis with conventional HPLC were analyzed with XLC-MS/MS and Deming regression analysis was applied. Concentrations ranged from 6.5-1500, 57-2200 and 170-9000 nmol/L for epinephrine, norepinephrine and dopamine, respectively. Reference intervals for urinary free catecholamines were obtained from the analysis of 24 h urine collections of 120 healthy subjects, participating in the LifeLines study[17]. LifeLines is a multi-disciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviour of 165,000 persons living in the North East region of The Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioural, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multimorbidity. In addition, the LifeLines project comprises a number of cross-sectional sub-studies which investigate specific age-related conditions. These include investigations into metabolic and hormonal diseases, including obesity, cardiovascular and renal diseases, pulmonary diseases and allergy, cognitive function and depression, and musculoskeletal conditions. Reference intervals were calculated using EP EvaluatorTM software[18] as recommended by the Clinical and Laboratory Standards Institute.

Table 2. Mass spectrometric parameters

Precursor m/z	Product m/z	Dwell time	Cone voltage	Collision energy
MRM Quantifiers				
184.15 (E)	166.10	0.105 sec	12 V	12 eV
169.15 (E-d3)	107.00	0.105 sec	32 V	18 eV
170.25 (NE)	152.00	0.105 sec	12 V	8 eV
176.25 (NE-d6)	158.00	0.105 sec	12 V	8 eV
154.15 (DA)	137.00	0.105 sec	17 V	17 eV
158.15 (DA-d4)	141.00	0.105 sec	17 V	17 eV
MRM Qualifiers				
184.15 (E)	166.10	0.105 sec	12 V	12 eV
187.15 (E-d3)	169.10	0.105 sec	12 V	12 eV
166.15 (E)	107.00	0.105 sec	32 V	18 eV
169.15 (E-d3)	107.00	0.105 sec	32 V	18 eV
152.15 (NE)	107.00	0.105 sec	25 V	18 eV
158.15 (NE-d6)	111.00	0.105 sec	25 V	18 eV
137.10 (DA)	91.00	0.105 sec	35 V	35 eV
141.10 (DA-d4)	95.00	0.105 sec	35 V	35 eV

Abbreviations: MRM: multiple reaction monitoring mode. m/z: mass to charge ratio. E: epinephrine, NE: norepinephrine, DA: dopamine, d3: 3 times deuterated compound, d6: 6 times deuterated compound, d4: 4 times deuterated compound.

Results

CHROMATOGRAPHY

Total cycle time per sample, including extraction, was 14 min. Epinephrine and its deuterated internal standard (E-d3) co-eluted after 7.3 min, norepinephrine and NE-d6 after 6.3 min and dopamine and DA-d4 after 8.7 min (Fig. 2). The identity of the compound was confirmed by the specific mass spectrum of an aqueous standard and a urine sample from a healthy subject (data not shown). No ion suppression was observed.

Detection limits

The limit of quantification (S/N 10:1) was 1.47 nmol/L for epinephrine, 15.8 nmol/L for norepinephrine and 11.7 nmol/L for dopamine with CVs of 9.0%, 6.2% and 11.8%, respectively (n=20).

Linearity and precision

Inter-assay linearity (n=18) obtained over a concentration ranges from 1.5-1500 nmol/L for epinephrine, 16-5000 nmol/L for norepinephrine and 12-9600 nmol/L for dopamine in oxidized blank urine was excellent. The mean slope was 0.0271 for epinephrine, 0.0016 for norepinephrine and 0.0008 for dopamine, with no significant intercept and correlation coefficients of 0.99. Aqueous calibration curves gave comparable slopes. Intra-assay precision was determined by replicate analyses in a single run at three concentrations (n=20). Inter-assay was determined by analysis of three concentrations over 4 weeks (n=18). For low concentrations intra-assay CVs were <10%. For medium and high concentrations intra-assay CVs were <2%. Inter-assay CVs were <10% for low concentrations and <5% for medium and high concentrations. Samples with high catecholamine concentrations exceeding the calibration ranges can be diluted up to 50 times, which results in comparable outcomes. Cartridges can be reused 2 times with consistent results and without occurrence of carry-over (< 0.1%).

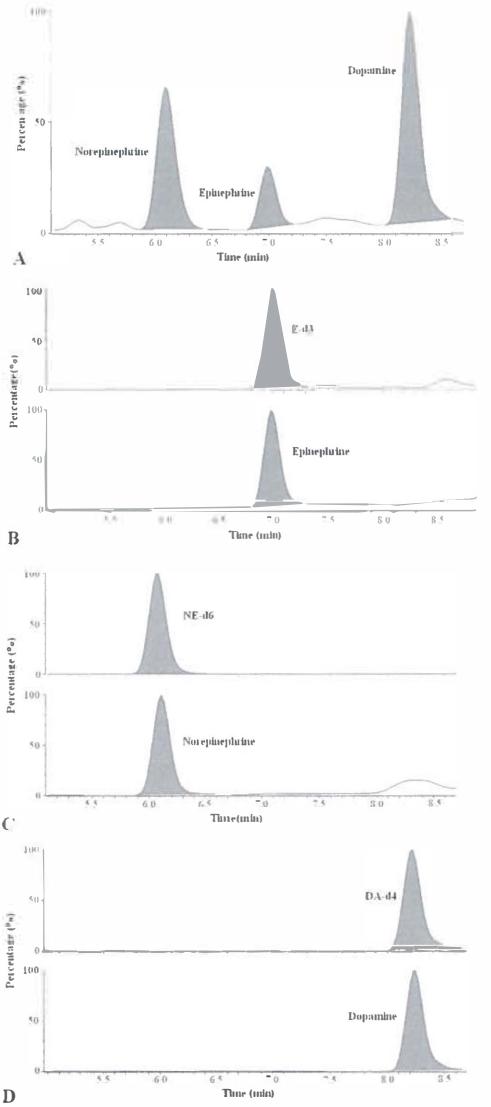


Fig. 2: Total ion current mass chromatograms and mass specific chromatograms of catecholamines and their deuterated internal standards in obtained in multiple reaction monitoring (MRM) mode by XLC-MS/MS for a urine sample from a healthy subject.

A: Total ion current mass chromatograms of all MRM transitions used. Retention times are 7.0, 6.1 and 8.2 min for epinephrine, norepinephrine and dopamine, respectively; B: Mass specific chromatograms of epinephrine and its deuterated internal standard E-d3 (m/z 184→166 and 169→107, respectively); C: Mass specific chromatograms of norepinephrine and its deuterated internal standard NE-d6 (m/z 170→152 and 176→158, respectively); D: Mass specific chromatogram of dopamine and its deuterated internal standard DA-d4 (m/z 154→137 and 158→141, respectively).

Retention time is indicated in min. Peak abundance is normalized to percentages relative to the highest peak in the chromatogram.

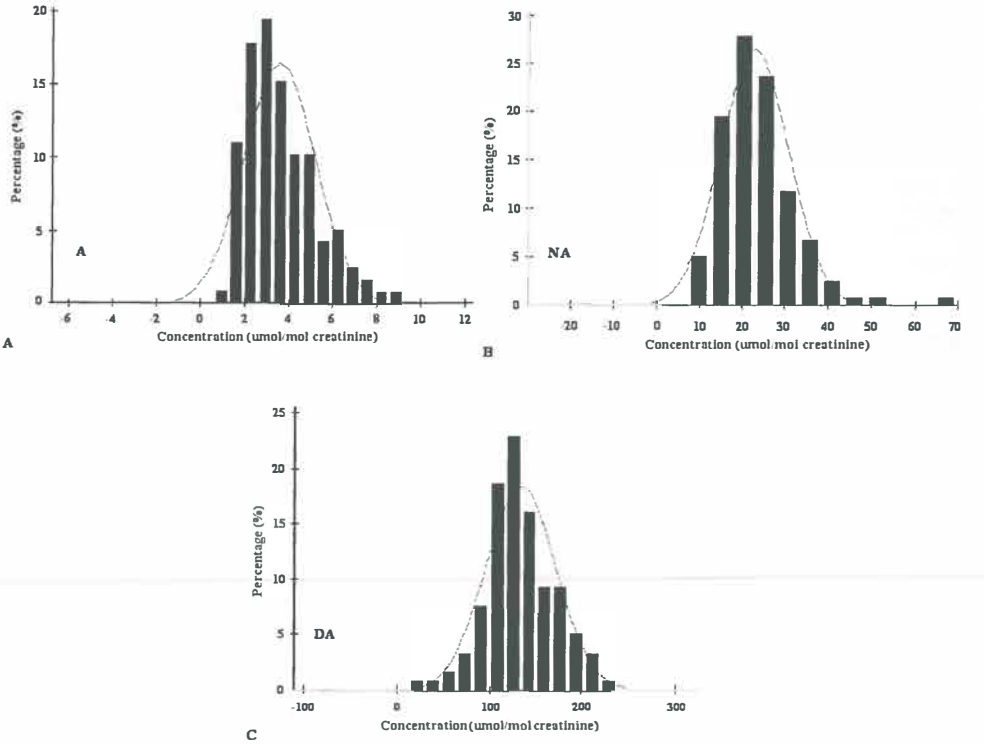


Fig. 3: Distribution of urinary catecholamine concentrations in reference samples from 120 healthy individuals in µmol/mol creatinine. The distributions of individual catecholamine concentrations are shown in histograms with a mean concentrations of 3.6, 23.5, 133.8 µmol/mol creatinine for epinephrine, norepinephrine and dopamine respectively. The black line shows a normal distribution which is in correspondence with the distribution of the individual serotonin concentrations. A: Epinephrine, reference interval 0-10 µmol/mol creatinine; B: Norepinephrine, reference interval 0-50 µmol/mol creatinine; C: Dopamine, reference interval 0-250 µmol/mol creatinine.

STABILITY

Urine samples, containing conservatives as described above, were found to be stable during 3 freeze-thaw cycles. In addition, isotope-diluted samples containing ascorbic acid were stable up to 9 days at 4-10 °C in the dark (autosampler).

URINE SAMPLES

The new XLC-MS/MS method correlated with the former HPLC with a correlation coefficient of 0.99 for epinephrine, 0.98 for norepinephrine and dopamine. The regression equations for the XLC-MS/MS method (x) and the HPLC method (y) had slopes of 1.15, 1.00 and 0.86 for epinephrine, norepinephrine and dopamine, respectively.

The distribution of urinary dopamine concentrations in the 120 healthy subjects for determination of the reference limits was normal and dopamine reference intervals were calculated with EP evaluator in parametric manner (Fig. 3A) according to CLSI C28-A2[23]. Concentrations of norepinephrine and epinephrine were logarithmically transformed in order to achieve normal distribution and these reference intervals were therefore calculated in a transformed parametric manner (Fig. 3B&C). Reference intervals in urine were 1-10, 10-50 and 60-225 µmol/mol creatinine for epinephrine, norepinephrine and dopamine, respectively.

Discussion

This study shows that physiological concentrations of catecholamines can be measured reproducibly and accurately in urine without extensive manual sample preparation and relatively short chromatographic time using automated on-line XLC-MS/MS. Importantly, due to tandem mass spectrometric detection chance of analytical interferences is theoretically diminished, which is another major drawback of conventional HPLC methods[11]. Catecholamines are biogenic amines characterized by an amino group in the alkalic side chain together with a phenolic catechol group (i.e. hydroxyl groups at 3- and 4-positions). The use of PBA-based extraction specific for catechol-containing compounds such as these catecholamines has been reported to be more effective than the commonly used SPE techniques as cation-exchange and activated alumina[20,21] for sample clean-up. The specificity of PBA-base extraction is based on the affinity of catecholamines for cartridge-bound PBA. In alkaline conditions (pH>8) formation of the reactive phenylboronate ion [PhB(OH)2-] is promoted. Compounds containing a catechol-group (cis-diols) covalently bind to the boronate ion on the cartridge by releasing a water molecule. The required alkaline conditions (pH>8) for formation of the reactive phenylboronate ion do not cause oxidation of the catecholamines, since this pH is only applied during extraction. The catecholamines are eluted from the PBA-catecholamine complex by applying an acidic solution[11], which is compatible with LC-MS/MS. On-line extraction with PBA-cartridges has not been performed previously for quantification of catecholamines in urine samples, although such method has been described for brain tissues [22].

Chromatographic separation of the three catecholamines is usually performed by reversed-phase LC with the use of expensive ion-pairing reagents[7,23,24]. Because of the highly polar nature of these biogenic amines such methods are required to achieve sufficient retention and separation. However, chromatographic runs are laborious while coupling to MS/MS is not possible due to incompatibility of the ion-pairing reagents with the electrospray ionization, resulting in signal suppression and MS contamination. Thus, for the

development of an XLC-MS/MS method other approaches of reversed phase chromatography are desired. The solution was found in a PFP propyl column, known to increase retention for compounds with electrophilic properties, as the catecholamines that are protonated on the amine-group under acidic conditions. In order to achieve retention of the catecholamines and especially NE, high percentages of aqueous mobile phase were required. Although the mass spectrometer prefers high organic contents for ionization, the sensitivity of the method showed to be excellent.

The chromatographic analysis time of the developed XLC-MS/MS method is relatively long in order to achieve complete separation of the three catecholamines and interfering compounds. In comparison with the previously used HPLC-ECD method at our laboratory, analysis time is greatly reduced from 8 hr sample preparation for a small sample batch and 30 min chromatography per sample to 14 min total analysis time per sample. Application of ultra performance liquid chromatography (UPLC) will most likely decrease chromatographic time further[25,26]. However, the on-line combination of UPLC with applied automated SPE system is not available yet.

Conclusions

We present a method for the selective automated analysis of free catecholamines in urine that uses on-line SPE coupled to LC-MS/MS. The advantages of the method include its high selectivity by the PBA affinity of catecholamines and the high specificity and sensitivity by unique MS/MS fragmentation of catecholamines, resulting in less interferences. Automation substantially reduces total analysis time leading to high throughput. This method therefore has several advantages over other methods for the analysis of catecholamines reported previously for the diagnosis and treatment of neuroendocrine disorders, especially pheochromocytoma.

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Chapter 6

Elevated Urinary Free and Deconjugated Catecholamines after Consumption of a Catecholamine-Rich Diet

Wilhelmina HA de Jong
Wendy J Post
Michiel N Kerstens
Elisabeth GE de Vries
Ido P Kema

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Abstract

Background: The biochemical diagnosis of pheochromocytoma depends on demonstration of elevated levels of catecholamines (i.e. epinephrine, norepinephrine and dopamine) and their metabolites. Aim of this study was to determine the pre-analytical influence of a catecholamine-rich diet on urinary free and deconjugated catecholamines in healthy volunteers with a highly specific and sensitive analytical technique.

Methods: A cross-over study was performed in 27 healthy adults. Subjects consumed catecholamine-rich nuts and fruits at fixed times on one day (about 35 μmol dopamine and 1 μmol norepinephrine) and catecholamine-poor products on another day. Urine samples were collected at timed intervals before, during and after experimental and control interventions. Automated on-line sample preparation coupled to isotope-dilution mass spectrometry was applied for the measurement of urinary concentrations of free and deconjugated catecholamines.

Results: The catecholamine-rich diet had substantial effects on urinary excretions of deconjugated dopamine (up to 20-fold increases) and norepinephrine (up to 10-fold). Dietary catecholamines had less but significant effects on urinary excretion of free dopamine and norepinephrine (up to 1.5-fold increases). Outputs of urinary free and deconjugated epinephrine remained unaffected.

Conclusions: Urinary excretion of deconjugated norepinephrine and dopamine is strongly affected by consumption of catecholamine-rich food products, thereby increasing the likelihood of a false-positive test result during hormonal evaluation for pheochromocytoma. Measurement of deconjugated catecholamines should therefore preferably be avoided, in favour of measurement of urinary free catecholamines. In case of demonstrating increased urinary excretion of deconjugated norepinephrine and dopamine, repeated measurements are warranted with dietary restrictions prior to sample collection.

Introduction

The biochemical diagnosis of pheochromocytoma is based on demonstration of elevated concentrations of compounds in the catecholamine metabolic pathway, i.e. urinary and plasma fractionated catecholamines (norepinephrine, epinephrine, dopamine) and their O-methylated metabolites (metanephrines)[1,2]. Several authors recommend measurements of plasma metanephrines for the diagnosis of pheochromocytoma[3-5], while others advocate the plasma test in combination with urinary measurements of catecholamines[6,7]. Currently, quantitative assays applied are state of the art, well-performing and insensitive to analytical interferences. However, biochemical results may be affected by pre-analytical factors, such as physiological influences (e.g., exercise, posture, stress) and medications (e.g., catecholamine reuptake blockers) that alter the production or disposition of catecholamines. We have demonstrated before that consumption of foods containing substantial quantities of biogenic amines (i.e. fruits and nuts) increase levels of urinary and plasma metanephrines[8].

The main fraction of catecholamines is converted to sulfate-conjugates by a sulfotransferase isoenzyme (SULT1A3), located in the gastrointestinal tract, that inactivates both endogenous and dietary-derived (exogenous) catecholamines[9-11]. Therefore, catecholamine outputs can be presented as free or deconjugated (sum of free and sulfate-conjugated) concentrations. Aim of this study was to examine the potential pre-analytical influence of a catecholamine-rich diet on urinary free and sulfate-conjugated catecholamines in healthy volunteers. Previous studies on this subject[12] were unable to distinguish between analytical interferences and physiological influences. Therefore, by application of an analytically state of the art automated isotope-dilution mass spectrometric method, this study for first time has been performed interference-free.

Materials and Methods

Analytical

Automated on-line sample preparation coupled to isotope-dilution liquid chromatography-mass spectrometry was applied for the measurement of urinary concentrations of free and deconjugated epinephrine, norepinephrine and dopamine. Immediately after collection, urine samples were acidified to pH 4 and preservatives ascorbic acid and ethylenediaminetetraacetic acid (EDTA) were added. Urine samples were diluted with deuterated internal standard and ascorbic acid solution (400 mg/L) and partly injected into the analysis system, equivalent to 5 µL of urine.

DIET EXPERIMENT

Subjects included 27 healthy adults (14 women, 13 men; median age 38 years, range 21-59 years) who served as their own controls by participating in both control and experimental arms of the protocol following a cross-over design, with at least one week between the randomly distributed test days. Both arms were preceded by an overnight fast from at least midnight until 08.30 h. Subjects avoided catecholamine-containing products (e.g., fruits, fruit drinks, nuts, potatoes, tomatoes and beans) the day before, during and the morning after both study days, as described previously[8].

For the experimental arm, all subjects consumed two catecholamine-rich meals. The first meal, consumed at 08.30 h, included 2-3 bananas (280 g of pulp), one-quarter of a fresh pineapple (185 g of pulp), 50 g shelled walnuts and 140 mL pineapple juice, purchased at local commercial outlets. At 10.30 h each subject drank 280 mL pineapple juice. At 12.30 h they consumed a second meal similar to the first. Finally, at 14.30 h, participants drank 280 mL pineapple juice. Based on previous measurements[13], total dopamine and norepinephrine intakes were respectively estimated at 35 μmol and 1 μmol; epinephrine was undetectable.

For the control arm, the subjects consumed meals (bread), snacks (gingerbread) and drinks (coffee, tea, dairy products) in accordance with the time schedule of the experimental arm. Morning urine until 08.30 h was gathered from all subjects. Four separate urine samples were then collected at 2 h intervals starting at 10.30 h with the fourth collection at 16.30 h. A final urine specimen was collected beginning at 16.30 h and ending at 08.00 h the next morning. Aliquots were stored within a day at -20 °C until analysis. The study was approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

DATA ANALYSIS

Results from the influence of diet on the free and total catecholamine levels are shown as mean values with 95% confidence intervals. First morning urine samples (until 08.30 h) served as reference points for dietary-associated changes over time. Linear mixed models, tested for significance at $P<0.05$, were used to determine the significance of temporal changes in analyte concentrations[14]. The model fit was evaluated for deviance and performed using the statistic software program ML Win version 2.0.2 (Centre for Multilevel Modelling, Bristol, UK). Time was included as a factor (fixed and random). The magnitude of a difference between the control and the experimental groups in this modeling is given by the interaction term between diet and time, since no differences at baseline concentrations between both groups were expected.

Results

URINARY DOPAMINE

Outputs of free and deconjugated dopamine were higher after ingestion of the high-catecholamine meals compared with control meals ($P<0.05$). After consumption of the second catecholamine-rich meal, outputs of free dopamine (Fig. 3A) were 1.5-fold ($P<0.05$) and deconjugated dopamine more than 20-fold higher ($P<0.05$) than baseline values (Fig. 3B). Free dopamine returned to baseline values overnight, whereas deconjugated dopamine remained elevated, but decreased significantly from values of the preceding collection. No significant changes in concentrations of free or deconjugated dopamine were apparent after ingestion of the control meals.

URINARY NOREPINEPHRINE

Free norepinephrine showed daytime increases and nighttime decreases ($P<0.05$) during the 24-h period after both catecholamine-rich and control meals (Fig. 3C&D.). Free norepinephrine excretion increased significantly more after the second meal in the diet group than in the controls, and remained elevated overnight. In addition, deconjugated norepinephrine showed significant ($P<0.05$) increases after ingestion of control vs. experimental meals up until the following morning (Fig. 3D). On the day of the catecholamine-rich diet, deconjugated norepinephrine increased by 10-fold over baseline values for the collection between 14.30 and 16.30 h and remained nearly 4-fold higher for the overnight collection.

URINARY EPINEPHRINE

Free and deconjugated epinephrine (Fig. 3E&F) showed the same time course of changes after control and experimental meals, indicating no influence of the catecholamine-rich diet. Deconjugated epinephrine, and to a lesser extent urinary free epinephrine, showed significant higher ($P<0.05$) excretions during daytime compared to night time.

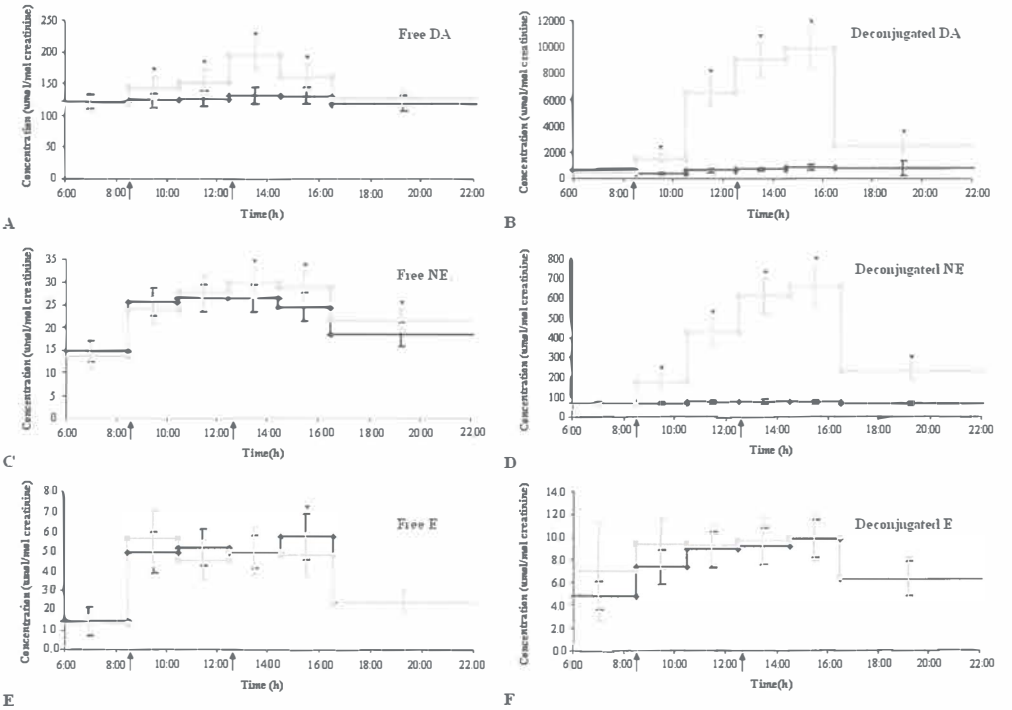

Fig. 1. Line graphs of mean urinary concentrations of catecholamines before, during and after catecholamine-rich and catecholamine-poor meals. Means were calculated from data of 27 subjects in both the control and the diet group and expressed with the 95% confidence intervals. Obtained statistic linear mixed models showed comparable graphs. Therefore, changes in concentrations in these models are significant ($P < 0.05$). * indicates significant differences between control and diet group data ($P < 0.05$).
A: urinary free dopamine; B: urinary deconjugated (free + sulfate-conjugated) dopamine; C: urinary free norepinephrine; D: urinary deconjugated norepinephrine; E: urinary free epinephrine; F: urinary deconjugated epinephrine.
Arrows indicate the times at which test meals were taken (8.30 h a.m. and 12.30 h). Grey line: significant model for the diet group (n=27); black line: significant model for the control group (n=27).
Abbreviations: E: epinephrine, NE: norepinephrine, DA: dopamine.

Discussion

The current study, performed with an analytically state of the art automated isotope-dilution mass spectrometric method, shows that diet composition substantially influences sulfate-conjugates of catecholamines present in urine. These findings are in agreement with our recent study on dietary influences of urinary and plasma metanephrines[9]. However, ingestion of catecholamine-rich foods has a more profound effect on urinary excretion of sulfate-conjugated catecholamines (up to 20-fold elevation) than on that of sulfate-conjugated metanephrines (up to 3-fold elevation)[9]. This is explained by the fact that ingested catecholamines are mainly converted to sulfate-conjugates in the gastrointestinal tract by SULT1A3[10-12], whereas only a small fraction is metabolized to metanephrines. Consequently, consumption of catecholamine-containing foods may significantly increase urinary excretion of sulfate-conjugated catecholamines[11,12], implicating diet restrictions prior to sampling should be established. In general, measurement of urinary deconjugated catecholamines has already been replaced by the measurement of urinary free catecholamines. In the present study, however, it is demonstrated that the free fractions of norepinephrine and dopamine are also significantly affected by diet composition, albeit to a substantial lesser extent than the deconjugated fractions. Therefore, diet restriction should also be considered preceding measurement of urinary free catecholamines. In addition, attention should be paid to the addition of acid preservatives against oxidation of catecholamines. Sulfate-conjugated catecholamines are sensitive to hydrolysis in an acid environment resulting in increased levels of the free fraction[16]. This will be especially important when conjugated levels are relatively more elevated than free levels due to dietary composition.

Reference values were not exceeded after consumption of the catecholamine-rich food products. However, the amounts of catecholamines consumed by our subjects were within or even lower than the amounts that might be expected in a usual Western diet. More specifically, Eldrup et al[17] investigated the influence of ordinary meals on plasma concentrations of sulfate conjugated catecholamines with one of the "ordinary" meals containing more than ten times the amount of catecholamines consumed in our study. In this context the doses of catecholamines consumed by our subjects do not appear unusual, which means higher doses have a risk of producing false positive results. In addition, catecholamines are present in a large variety of foods (other than fruits and nuts[8]) in amounts that have not been precisely delineated, such as tomatoes, beans, cheeses, fermented foods and processed meat products. Besides, dietary influences may not be confined to food products that contain catecholamines[18], as we discussed previously[9].

Therefore, it is extremely difficult to provide a complete list of foods that can be consumed in a restricted diet.

Only a few studies investigated the influences of consumed catecholamines on urinary catecholamine excretion. These studies were all performed at least 20 year ago, which underlines the importance of the present study to examine this issue with analytically state-of-the art equipment. Our results are in correspondence with two of those previous studies that investigated the consumption of bananas[13,19] which also demonstrated a rise in urinary deconjugated and, to a lesser extent, in free catecholamines. However, these results are in contrast to another study that
found no changes in urinary excretion of free catecholamines after consumption of catecholamine-rich foods[20]. This is probably explained by differences in the performance of applied analytical procedures. We used an automated mass spectrometric method with high analytical performance for the analysis of urinary catecholamines, which enabled us to quantify pre-analytical influences on the final test result with substantially improved accuracy. The most recent study, dating back to 1990, concluded that dietary restrictions prior to urinary catecholamine diagnostics were not necessary, because no differences were observed between urinary outcomes of a catecholamine-low diet and a 'normal' diet[21]. However, this study did not investigate the consumption of catecholamine-rich food products.

The present study also shows daytime increases and nighttime decreases in circulatory outputs of urinary free and deconjugated catecholamines, consistent with previous observations[22] likely reflecting increased sympathoadrenal outflow related to a more ambulatory and active status during waking hours. This biological variation emphasizes the need to collect 24-h urine samples instead of urine portions. Concurrently, effects of diet on the excretion of free catecholamines will be diminished due to dilution of the urine over the 24-h collection period.

In conclusion, urinary excretion of deconjugated catecholamines and, to a lesser extent, of free catecholamines, is affected by consumption of catecholamine-rich food products, thereby increasing the likelihood of false-positive biochemical test results for the diagnosis of pheochromocytoma. We therefore recommend the measurement of free catecholamines over deconjugated catecholamines, and suggest repeated measurement of urinary free catecholamines in order to confirm the presence of an increased urinary catecholamine excretion. Dietary restrictions 24 hrs prior to this repeated sampling should be sufficient to circumvent increased levels by consumption of catecholamine-containing foods. After all,

this study showed that overnight catecholamine levels are returning to normal. Alternatively, one could measure plasma free metanephrines, which are not affected by dietary catecholamines[9]. Thus, the results of our study further strengthen the recommendation of plasma free metanephrines as the biochemical test of choice for the diagnosis of pheochromocytoma[3].

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PART II

Tryptophan metabolism

Chapter 7

Urinary 5-HIAA measurement using automated on-line solid-phase extraction-high-performance liquid chromatography-tandem mass spectrometry

Wilhelmina HA de Jong
Kendon S Graham
Elisabeth GE de Vries
Ido P Kema

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Abstract

Quantification of 5-hydroxyindole-3-acetic acid (5-HIAA) in urine is useful for diagnosis and follow-up of patients with carcinoid tumors and for monitoring serotonin (5-hydroxytryptamine) metabolism in various disorders. We describe an automated method (XLC–MS/MS) that incorporates on-line solid-phase extraction (SPE), high-performance liquid chromatography (HPLC) and tandem mass spectrometric (MS/MS) detection to measure urinary 5-HIAA. Automated pre-purification of urine was carried out with HySphere-Resin GP® SPE cartridges containing strong hydrophobic polystyrene resin. The analyte (5-HIAA) and internal standard (isotope-labelled 5-HIAA-d_2) were, after elution from the cartridge, separated by reversed-phase HPLC and detected with tandem MS. Total cycle time was 5 min. 5-HIAA and its deuterated internal standard (5-HIAA-d_2) were retained on and eluted from the SPE cartridges in high yields (81.5–98.0%). Absolute recovery was 96.5–99.6%. Intra-assay (n=20) and inter-assay (n=20) CVs for the measurement of 5-HIAA in urine in three concentration levels ranged from 0.8 to 1.4% and 1.7 to 4.2%, respectively. For urine samples from patients (n=78) with known or suspected metastatic carcinoid tumors, results obtained by XLC–MS/MS were highly correlated (R^2=0.99) with the routinely used fluorometric method. This XLC–MS/MS method demonstrated lower imprecision and time per analysis (high-throughput) than manual solvent extraction methods and higher sensitivity and specificity than non-mass spectrometric detection techniques.

Introduction

5-Hydroxyindole-3-acetic acid (5-HIAA) is the most abundant metabolite of serotonin (5-hydroxytryptamine: 5-HT)[1]. The neurotransmitter/neurohormone serotonin is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut and in serotonergic neurons in the central nervous system[2,3]. Peripheral serotonin is metabolized mainly in the lung and the liver through enzymatic conversion by monoamine oxidase-A (MAO-A; EC 1.4.3.4), resulting in urinary excretion of 5-HIAA. Serotonin plays an important role in carcinoid syndrome[4,5]. Therefore, quantification of urinary 5-HIAA is especially important in the diagnosis and follow-up of carcinoid patients.

Furthermore serotonin is hypothesized to be involved in schizophrenia, depression, migraine and autism[4,6,7], while 5-HIAA is increased in Whipple disease, celiac disease and tropical spruce. Additionally, urinary 5-HIAA can be influenced by the diet serotonin content[8].

Today, analytical methods have been described to measure 5-HIAA in urine, including immunoassays, gas chromatography and liquid chromatography coupled to several detection techniques[9]. These methods may suffer from interferences and are time consuming, because of necessary sample clean-up.

We developed an automated on-line solid-phase extraction-liquid chromatographic method with tandem mass spectrometric detection (XLC–MS/MS) for the measurement of urinary 5-HIAA. This method combines the best of two previously described methods: selective and specific liquid chromatographic–tandem mass spectrometry[10,11] and on-line solid-phase extraction (SPE) coupled to high-performance liquid chromatography (HPLC) with fluorometric detection[12]. XLC–MS/MS is in our laboratory considered as a promising method for several applications[13].

Materials and Methods

Chemicals and reagents

HPLC-grade acetonitrile was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland); formic acid 98–100% ultrapure from BDH Laboratory Supplies (Poole, UK) and ammonium formate 99.995^{+}% from Sigma–Aldrich Ltd. (Steinheim, Germany). 5-HIAA was purchased from Sigma–Aldrich Ltd. (Steinheim, Germany) and 5-HIAA-d_2 from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada); urine preservatives ascorbic acid and EDTA were from Merck KGaA (Darmstadt, Germany). Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure. All chemicals and solvents were of analytical reagent grade.

INSTRUMENTS

A Spark Holland Symbiosis® on-line SPE system (Spark Holland, Emmen, the Netherlands) was used for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (two high-pressure dispensers), and an HPLC pump, as shown in Fig. 1. The ACE module contains two connectable six-way valves and a SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and clean-up. The integrated HPLC pump was a binary high-pressure gradient pump. Column temperature was controlled with a Mistral Column Oven (Spark, Holland). Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters, Milford, MA). All aspects of system operation and data acquisition were controlled using MassLynx V4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

SAMPLE PREPARATION

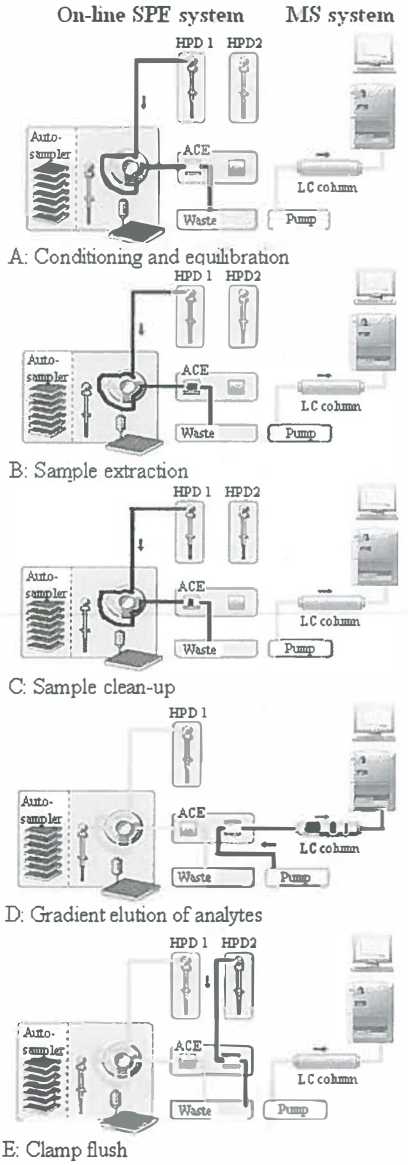
Stock solutions of 5-HIAA and 5-HIAA-d_2 (1 g/L) were prepared in aqueous formic acid (0.1 mol/L). Working solutions were water diluted from the stock solutions. Calibrators were prepared in water by addition of working solution corresponding to concentrations of 0.0, 6.6, 13.2, 26.5, 66.2, 198.5, 529.3 and 1191.0 µmol/L 5-HIAA.

Fifty microliters of urine (acidified to pH 4 and containing the conservatives ascorbic acid and EDTA, added prior to collection) were mixed directly in an autosampler vial with 100 µL internal standard solution (1 µg). After dilution with 850 µL water, 50 µL of each sample was injected into the XLC–MS/MS system. This injection volume was equivalent to 2.5 µL of urine.

For method-comparison studies, urine samples were used from 78 patients with suspected or known metastatic carcinoid tumors of the mid-gut which already had been measured with the routinely used HPLC-fluorometric method[12].

ON-LINE SPE

Sample clean-up took place by on-line SPE, following a similar procedure as described previously[12,13]. HySphere-Resin GP® 10 mm × 2 mm cartridges (Spark, Holland) were used for sample extraction. The Symbiosis® system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted by solvents delivered by two high pressure dispensers (HPD1 and HPD2). Both HPD's have several ports connected to different solvents.



A: Conditioning and equilibration

A1: New cartridge	From tray to left clamp
A2: Conditioning	Acetonitrile (1 mL at 2 mL/min)
A3: Equilibration	Water (2 mL at 2 mL/min)

B: Sample extraction

B1: Injection	Start loop fill with sample
B2: Extraction	Sample loading using water (500 µL at 500 µL/min)

C: Sample clean-up

C1: Wash cartridge	Water (1 mL at 2.5 mL/min)
C2: Wash cartridge	Ammoniumformatebuffer 10 mmol/L pH 3 (1 mL at 2 mL/min)

D: Gradient elution of analytes

D1: Transfer cartridge	From left to right clamp
D2: Elution	Elution of analytes from cartridge to LC column using LC gradient (1.0 min)
D3: New cartridge	From tray to left clamp

E: Clamp flush

E1: Clamp flush	Water (2 mL at 1 mL/min)
E2: Clamp flush	Acetonitrile (2 mL at 2 mL/min)
E3: Move cartridge	From right clamp to tray

Figure 1. Schematic representation of the Symbiosis® on-line SPE system coupled to HPLC with mass spectrometric detection (XLC–MS/MS). *A*: Conditioning and activation of the SPE cartridge in the left clamp. *B*: Sample extraction after filling of the autosampler loop. *C*: Sample clean-up. *D*: Right clamp gradient elution of analytes with LC mobile phase, directly followed by chromatographic separation and mass spectrometric detection. *E*: Cartridge and clamp clean-up. HPD: high-pressure dispenser.

The analytes were eluted directly on the analytical column, as is schematically shown in Fig. 1. The cartridges, located in the left clamp, were conditioned and equilibrated with acetonitrile and water (Fig. 1A), respectively. Subsequently, the sample was loaded on the cartridge with water (B) and wash steps were performed with water and 10 mM ammoniumformate pH 3 (C). After cartridge transfer to the right clamp, the analytes were eluted from the cartridge by LC gradient elution (200 µL of mobile phase; D). After elution, and during chromatography, the cartridge was regenerated in the right clamp by clamp flushes with water and acetonitrile (E). Processing of subsequent samples was carried out in parallel.

Liquid chromatography

Chromatographic separation was achieved by using a reversed-phase Atlantis dC18 HPLC column (3 mm × 100 mm I.D.; 3 µm; Waters). A gradient flow of 0.2% aqueous formic acid (A) and acetonitrile (B) (flow rate 0.40 mL/min) was applied to the chromatographic column as shown in Table 1. Total cycle time per sample was 5 min. 5-HIAA and its deuterated internal standard co-eluted after 2.5 min. Gradients applied were linear. Column temperature was kept at 25 °C.

Mass spectrometry

The mass spectrometer was directly coupled to the chromatographic column. In positive electrospray ionization mode 5-HIAA and its deuterated internal standard were protonated to produce ions at the form $[M+H]^+$, with *m/z* 192 and *m/z* 194, respectively. Upon collision-induced dissociation (CID) with Argon gas, these precursor ions produced characteristic product ions of *m/z* 146 [M–COOH] and 117 [M–C_2COOH] for 5-HIAA and *m/z* 148 and 119 for the deuterated internal standard. A multiple reaction-monitoring mode (MRM) was developed for the specific *m/z* transitions 192→146 and 192→117 (5-HIAA) and 194→148 and 194→119 (internal standard) using a dwell time of 0.1 s and an interchannel delay of 10 ms. The second mass transition was used as qualifier, following EU directive 2002/657/EC. Main mass spectrometric parameters are shown in Table 2. Ion suppression was not observed when 5-HIAA and its deuterated internal standard were infused constantly during injection of blank samples and urine samples.

Table 1: LC gradient parameters.

Time	Flow (mL/min)	% A 0.2% Formic Acid	% B Acetonitrile
0:00	0.40	70	30
1:00	0.40	70	30
4:00	0.40	95	5
4:30	0.40	70	30
5:00	0.40	70	30

Table 2: Main working parameters for tandem mass spectrometry.

MS part	Parameter	Value
Source	Ion mode	ESI+
	Capillary voltage	4 kV
	Cone voltage	17 V
	Extractor voltage	4 V
	RF lens voltage	0.2 V
	Source temperature	150°C
	Desolvation temperature	450°C
	Desolvation gas flow	1000 L/h
	Cone gas flow	50 L/h
Collision cell	Argon flow	0.4 mL/min
	Collision energy	17 eV

Results

CHROMATOGRAPHY

Total sample analysis time, including extraction, was 5 min. A deuterated internal standard was used, since the mass spectrometer monitored precursor as well as product ions with high analytical specificity. The identity of the compound was confirmed by the specific mass spectrum. In Fig. 2 mass chromatograms are shown of a standard (A), a patient sample with normal 5-HIAA concentration (B) and a carcinoid patient sample with elevated 5-HIAA concentration (C). An additional peak from an unknown compound can appear in chromatograms near to the 5-HIAA peaks. This peak is present in most patients and healthy controls. In some samples, the extra peak is not visible because of the significant difference in concentration with respect to 5-HIAA (C).

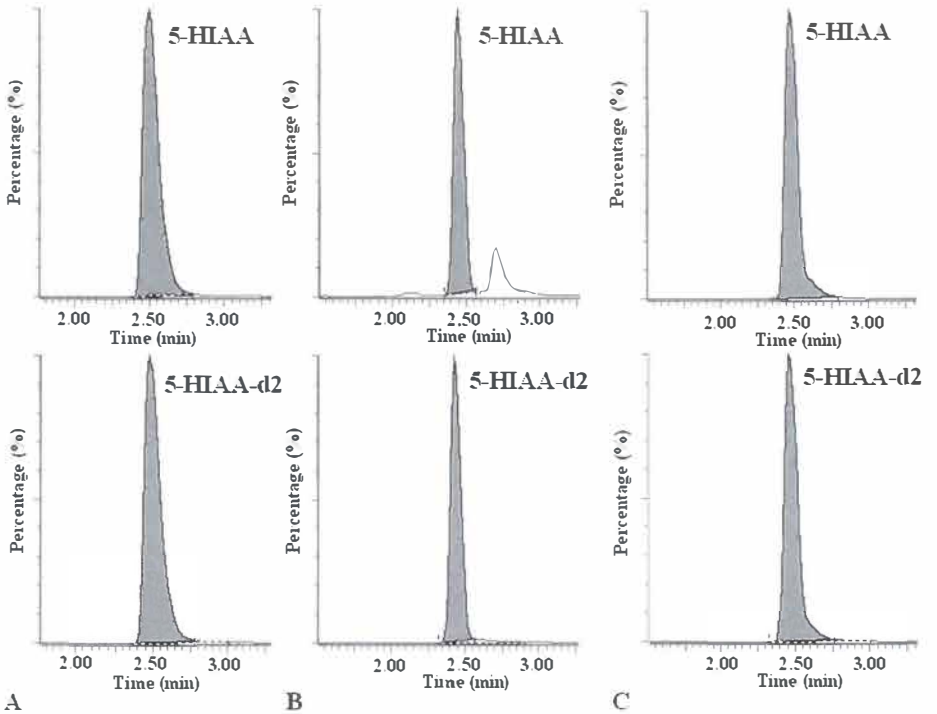
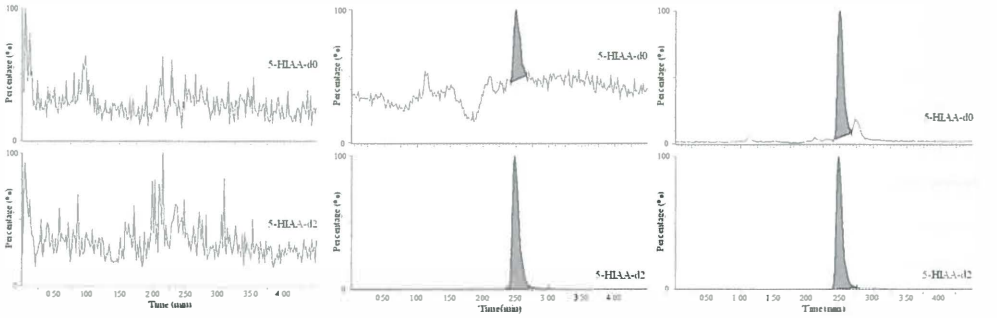


Figure 2. Chromatograms of 5-HIAA in an aqueous calibrator and two urine samples, obtained by XLC–MS/MS analysis. Retention time of 5-HIAA is 2.5 min. *A*: Aqueous standard containing 66.17 µmol/L 5-HIAA, d_0 area 373034, d_2 area 474911. *B*: Urinary patient sample with low concentration of 5-HIAA (6.53 µmol/L), d_0 area 31921, d_2 area 390900. *C*: Urinary patient sample with high concentration of 5-HIAA (125.09 µmol/L), d_0 area 495205, d_2 area 351582.

DETECTION LIMITS

The lower limit of detection (LLOD), defined as the minimum signal-to-noise ratio of at least 3:1 was <0.10 µmol/L. The limit of quantification (S/N 10:1) was 0.13 µmol/L with a CV of 9.5% (n=20). Chromatograms of a blank sample (A) and of LLOD (B) and LLOQ (C) are shown in Fig. 3. LLOQ and LLOD are both diluted urine samples (healthy controls), which explains the fact that in these samples the additional peak of the unknown compound occurs.



A B C

Figure 3. Chromatograms of an aqueous blank sample and of urine samples at LLOD and LLOQ. Retention time of 5-HIAA is 2.5 min. Precursor product transitions of 191.90→146.15 (d_0) and 193.90→148.15 (d_2) are shown. *A*: Aqueous blank sample. *B*: Urine sample at LLOD (<0.10 µmol/L), d_0 area 445, d_2 area 799745. *C*: Urine sample at LLOQ (0.13 µmol/L), d_0 area 6257, d_2 area 754968.

Table 3: Precision of XLC-MS/MS method.

	Concentration (SD) (µmol/L)	CV (%)
LOD	< 0.10	
LOQ (*n*=20)	0.13	9.48
Intra-assay (*n*=20)		
Low	13.7 (0.1)	0.91
Medium	257.2 (3.6)	1.39
High	989.1 (7.7)	0.78
Inter-assay (*n*=20)		
Low	13.6 (0.7)	4.93
Medium	252.2 (9.2)	3.63
High	968.6 (21.5)	2.22

Abbreviations: LOD: limit of detection; LOQ: limit of quantification; SD: standard deviation; CV: variation coefficient.

LINEARITY AND PRECISION

The inter-assay linearity (n=8) obtained over a concentration range from 0 to 1200 µmol/L 5-HIAA was excellent. The mean slope was 0.0102, intercept was 0.004 µmol/L and correlation coefficient was 0.9996. Spiked urine calibration curve gave a comparable slope (0.0098), but a different intercept (0.17 µmol/L), because of the endogen basal 5-HIAA concentration present in pooled urine, which confirms the selectivity of the method.

Intra-assay precision was determined by replicate analyses in a single run at three concentrations (n=20). Inter-assay was determined by analysis of three concentrations over 8 weeks (n=20). For all concentrations CV was found to be <5%. Intra-assay CV (n=20) was 0.8–1.4%. Inter-assay CV (n=20) was 2.2–4.9%. Precision data are shown in Table 3. Plasma samples with high 5-HIAA concentrations that exceed the calibration range can be diluted up to 100 times.

Table 4: Stability of 5-HIAA in urine.

	Concentration (µmol/L)		
Freeze-thaw (n=3)	**0x**	**3x**	
Low	14.1	14.2	
Medium	253.8	266.2	
High	997.5	1003.2	
	0 hr	**24 hr**	**48 hr**
Stability 4 °C			
Low	14.1	14.0	13.6
Medium	253.8	254.9	259.2
High	997.5	989.3	983.4
Stability 10 °C			
Low	14.1	14.3	13.6
Medium	253.8	260.6	254.5
High	997.5	980.1	982.6
Stability room temperature			
Low	14.1	14.1	13.6
Medium	253.8	260.2	256.9
High	997.5	982.4	997.9

RECOVERY AND STABILITY

Recovery experiments were conducted with spiked standard addition in three concentrations (low, medium and high) measured with and without solid-phase extraction. Recoveries ranged from 96.5 to 99.6%. Absolute recovery on the SPE cartridge was measured with spiked urine samples in the same concentration levels. These recoveries ranged from 81.5 to 98.0%. Samples, containing conservatives as described above, were found to be stable during three freeze–thaw cycles and for 48 h at 4 °C, 10 °C (autosampler) and at room temperature. Stability results are shown in Table 4. Consistent results were obtained of repeated sample measurement on the same cartridges (n=30), without occurrence of carry-over (<0.1%).

METHOD COMPARISON

For method comparison 78 patient samples, routinely analyzed in our laboratory for 5-HIAA by on-line SPE coupled to HPLC with fluorometric detection with a concentration range up to 60 mmol/mol creatinine, were reanalyzed with the new XLC–MS/MS method. The regression equation (according to Deming regression analysis) for the XLC–MS/MS method (x) and the HPLC method (y) had a slope of 1.251 and an intercept of −4.458 µmol with a correlation coefficient of 0.99. Graphs are shown in Fig. 4.

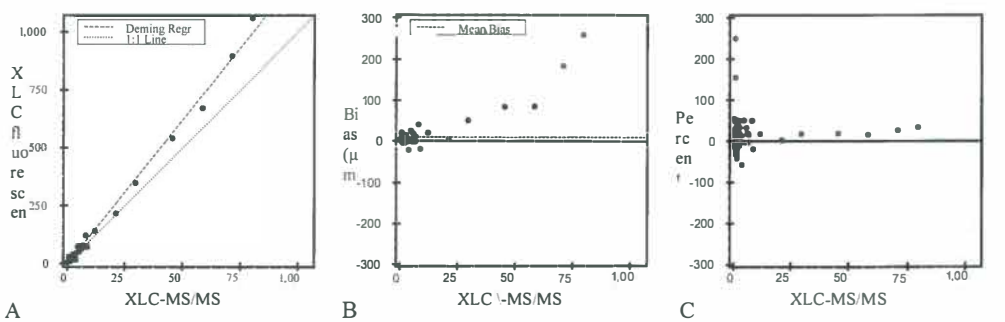


Figure 4. Correlation between the new (x: XLC–MS/MS) and the old (y: HPLC-fluorometric detection) method for the measurement of 5-HIAA in urine. *A*: Deming regression scatter plot; equation $y = 1.251x - 4.458$. *B*: Bias plot. *C*: Percentage bias plot.

Discussion

An automated on-line solid-phase extraction-liquid chromatographic method with tandem mass spectrometric detection (XLC–MS/MS) for the measurement of urinary 5-HIAA was developed by combining previously described methods[10-12]. The current method uses the best characteristics of these methods: selective and specific detection with tandem mass spectrometry and automated on-line sample clean-up.

Routinely, we already used an automated on-line sample procedure with fluorometric detection[12] for the quantification of 5-HIAA in urine. Automated sample preparation reduces sample time and intra- and inter-assay variation, which increases laboratory efficiency and accuracy. However, major limitations of this HPLC method include the long analysis time (19 min per sample), the use of a non-isotope labelled internal standard (5-HICA) and the biased results for high concentrated samples. In addition, the detection method could be improved by the use of mass spectrometry. MS/MS detection is more specific because of the selection of the precursor to product mass transition, which enables 'simple' identification of the analyte. Furthermore MS/MS allows the use of a stable isotope-labelled internal standard, while time for chromatographic separation can be reduced.

SPE was directly coupled to the HPLC, as described before[12]. Retention of 5-HIAA on SPE cartridges was based on hydrophobic interaction between the analytes and the sorbent. By increasing the organic phase in the elution step, analytes were released from the cartridge. The use of anion exchange cartridges did not improve analyte extraction at different pH, possibly due to the amphoteric nature of 5-HIAA, as became clear after research for the best cartridge material.

Urine was acidified after collection for conservation by preventing ionization of the carboxyl group and to facilitate retention of 5-HIAA on the reversed-phase SPE stationary matrix. Furthermore, manual sample preparation consisted of a 20-fold dilution in order to reduce ion suppression during the mass spectrometric analysis and addition of the deuterated internal standard.

During method development the additional peak of the unknown compound in the chromatogram was not an issue, since it was well separated from the 5-HIAA peak. However, after 2 months of column use for routine sample measurement, column characteristics changed. The peak from the unknown compound came nearer to the 5-HIAA peak, which made it more difficult to quantify 5-HIAA. To improve resolution between the two compounds, the column was replaced by a longer column (Atlantis dC18; 3.0 mm × 150 mm; particle size 3 μm) to improve chromatographic separation of the two compounds. Retention time is thereby prolonged by 2 min.

Conclusion

We have developed a method for the routine determination of urinary 5-HIAA that overcomes the limitations of an existing on-line HPLC procedure. In particular, when using XLC–MS/MS, sample analysis time is considerably shorter (5 min versus 19 min), chance of chromatographic interferences is reduced and dilution of concentrated samples is not necessary because of the broad linear calibration range. Furthermore the method is more reliable because of the use of an isotope-labelled internal standard and the 5-HIAA-specific mass transitions measured. Finally, XLC–MS/MS is a promising method that enables automated, high-throughput, accurate quantification of several other clinical important biomarkers.

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Chapter 8*A*

Automated Mass Spectrometric Analysis of Urinary and Plasma Serotonin

Wilhelmina HA de Jong
Marianne HLI Wilkens
Elisabeth GE de Vries
Ido P Kema

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Abstract

Background: Serotonin emerges as crucial neurotransmitter and hormone in a growing number of different physiologic processes. Beside extensive serotonin production previously noted in patients with metastatic carcinoid tumors, serotonin now is implicated in liver cell regeneration and bone formation. The aim was to develop a rapid, sensitive and highly selective automated on-line solid-phase extraction method coupled to high performance liquid chromatography-tandem mass spectrometry (XLC-MS/MS) to quantify low serotonin concentrations in matrices such as platelet-poor plasma and urine.

Methods: Fifty µL plasma or 2.5 µL urine equivalent were pre-purified by automated on-line solid-phase extraction, using weak cation exchange. Chromatography of serotonin and its deuterated internal standard was performed with hydrophilic interaction chromatography. Mass spectrometric detection was operated in multiple reaction monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionisation. Serotonin concentrations were determined in platelet-poor plasma of metastatic carcinoid patients (n = 23) and healthy controls (n = 22). Urinary reference intervals were set by analyzing 24h-urine collections of 120 healthy subjects.

Results: Total run-time was 6 min. Intra- and inter-assay analytical variation were <10%. Linearity in the 0-7300 µmol/L calibration range was excellent (R^2>0.99). Quantification limits were 30 and 0.9 nmol/L in urine and plasma, respectively. Platelet-poor serotonin concentrations in metastatic carcinoid patients were significantly higher than in controls. The urinary reference interval was 10-78 µmol/mol creatinine.

Conclusions: Serotonin analysis with sensitive and specific XLC-MS/MS overcomes limitations of conventional HPLC. This enables accurate quantification of serotonin for both routine diagnostic procedures and research in serotonin-related disorders.

Introduction

Serotonin (5-HT: 5-hydroxytryptamine) is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut and in serotonergic neurons in the central nervous system. The major part of dietary tryptophan is used for protein synthesis, while about 2% of the dietary tryptophan is used for the serotonin production. Serotonin is stored predominantly (about 80%) in the enterochromaffin cells of the gastrointestinal tract, in the dense granules of platelets and in the serotonergic neurons of the central nervous system[1-3]. Peripheral serotonin is metabolized mainly in the lung and the liver via enzymatic conversion by monoamine oxidase-A (MAO-A; EC 1.4.3.4), resulting in urinary excretion of 5-hydroxyindole-3-acetic acid (5-HIAA)[3].

Serotonin is a neurotransmitter involved in CNS neurotransmission processes, but also emerges as a crucial hormone in a growing number of different physiologic processes outside the brain. Depletion of serotonin is implicated in liver cell regeneration[4], bone formation and psychiatric diseases[5-8], while extensive serotonin production has been noted in carcinoid tumors[5,9,10]. Depending on the site of origin, carcinoid tumors can give rise to excessive synthesis, storage, and release of serotonin; its precursor, 5-hydroxytryptophan (5-HTP); and its major metabolite, 5-HIAA. For the clinical chemical diagnosis and follow-up of patients with carcinoid tumors, measurements of serotonin, its precursors and its metabolites in body fluids and tissues are used.

In previous studies, we showed that platelet serotonin is the most accurate marker for the diagnosis of carcinoids[11,12]. In healthy subjects, but not in carcinoid patients 99% of circulatory serotonin is accumulated and stored in platelets. Therefore especially the measurement of the free serotonin concentration in platelet-poor plasma might provide information about the level of serotonin overproduction which is of clinical relevance for carcinoid patients, since the free fraction is the serotonin pool that actually binds to the serotonin receptor and exerts its actions.

Normally, circulating serotonin is almost entirely confined to platelets. Due to its low concentrations and the resulting analytical detectability problems, free plasma serotonin has not been measured routinely. 5-HIAA in urine is used for follow-up of carcinoid patients. It reflects best the tumor burden as it is the metabolic end product resulting from free and stored serotonin turnover. Urinary serotonin is less important, but is considered to reflect clearance of plasma free serotonin and renal decarboxylated 5-HTP[13,14]. Quantification can however be useful in the diagnosis of midgut and aromatic amino acid decarboxlyase (AADC)-deficient foregut carcinoids[9,11,13,15]. In previous studies it was noted that some of these tumors cannot produce serotonin, because of a deficiency in the enzyme AADC responsible for the conversion of 5-HTP to serotonin. In these cases, some 5-HTP can be decarboxylated by the kidney and thus excreted in the urine as serotonin. Urinary serotonin

measurement was therefore considered to be a useful marker in the diagnosis of AADC-deficient carcinoid[13].

Several analytical techniques have been developed for the measurement of serotonin and its related compounds. HPLC-based techniques are commonly used[12,16] and easily coupled to mass spectrometric detectors to increase analytical sensitivity and specificity and reduce the chance of interferences. In addition, automation of sample pre-treatment is emerging[17,18], since it enables the reduction of analytical variation attributable to differences in manual sample pretreatment and, moreover, decreases analysis turnaround time.
In order to enable quantification of serotonin in platelet-poor plasma and to replace the laborious, currently used method for urinary serotonin, we developed an automated on-line solid-phase extraction-liquid chromatographic method with tandem mass spectrometric detection (XLC-MS/MS) for the measurement of serotonin. This method combines selective and specific liquid chromatographic–tandem mass spectrometry with on-line solid-phase extraction (SPE) and uses similar analytical principles as previously described for plasma metanephrines analysis[19]. With this technique, we quantified plasma free serotonin in 22 healthy controls and 23 carcinoid patients and established the reference interval for urinary serotonin excretion in 24-h urine collections in 120 healthy subjects.

Experimental

Chemicals and reagents

HPLC-grade acetonitrile was obtained from Rathburn Chemicals Ltd. (Walkerburn, UK); ammonium formate 99.995^{+}% from Sigma-Aldrich Ltd. (Steinheim, Germany); formic acid 98% to 100% ultrapure from BDH Laboratory Supplies (Poole, UK); sodium hydroxide (NaOH), hydrochloric acid and urine preservatives ascorbic acid and EDTA were acquired from Merck KGaA (Darmstadt, Germany).
Serotonin was purchased from Sigma-Aldrich Ltd. and serotonine-α,α,ß,ß-d4 creatinine sulfate complex from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada); Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure. All chemicals and solvents were of analytical reagent grade.

Instruments

A Spark Holland Symbiosis® on-line SPE system (Spark Holland, Emmen, the Netherlands) was used for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10°C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (2 high-pressure dispensers), and an HPLC

pump, as shown previously[19,20]. The ACE module contains 2 connectable 6-way valves and a SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and cleanup. The integrated HPLC pump was a binary high-pressure gradient pump. Column temperature was controlled with a Mistral Column Oven (Spark Holland). Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters, Milford, MA). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

BIOLOGICAL SAMPLES

For method-comparison studies, urine samples with a concentration range up to 4240 µmol/L were used from 60 patients with suspected or known metastatic carcinoid tumors. The reference interval for urinary free serotonin was obtained from the analysis of 24 h urine collections of 120 healthy subjects, participating in the LifeLines study[21]. The reference interval was calculated with EP Evaluator™ software[22] as recommended by the Clinical and Laboratory Standards Institute (CLSI) according to CLSI C28-A2[23]. LifeLines is a multi-disciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviors of 165,000 persons living in the North East region of The Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multimorbidity. In addition, the LifeLines project comprises a number of cross-sectional sub-studies which investigate specific age-related conditions. These include investigations into metabolic and hormonal diseases, including obesity, cardiovascular and renal diseases, pulmonary diseases and allergy, cognitive function and depression, and musculoskeletal conditions.

Plasma free serotonin levels were determined in a prospective pilot study of 22 healthy controls and 15 patients with a metastatic serotonin producing carcinoid tumor[24], which was extended with 8 patients to a total of 23 carcinoid patients. Blood for platelet-poor plasma was obtained by venipuncture using a butterfly needle to collect in 5 mL plastic syringes containing 60 µL 7.5% K_3EDTA solution. Blood was centrifuged within 30 min after drawing at 12,000 g for 2 min in an Eppendorf Microcentrifuge at 22 °C[25]. The remaining plasma was stored at -80 °C until analysis. The studies were approved by the medical ethical committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

SAMPLE PREPARATION

Working solutions of serotonin were diluted from a freshly weighed stock solution (1 mg/mL) on the day of analysis. Working solutions of serotonin-d_4 (1 µM) were diluted from the stock solution (0.4 mg/mL in 0.04 M ascorbic acid) that was stored at -20 ºC for 6 months. Aqueous calibrators were prepared by addition of working solution corresponding to concentrations from 30 to 7300 nmol/L serotonin for urinary analysis and from 3.49 to 4900 nmol/L for plasma. All solutions were prepared in water with ascorbic acid added as preservative (400 mg/L). Urinary calibration curves were aqueous. Plasma calibration curves were made using blank plasma, obtained by dialysis. Low, medium and high quality-control samples were made by pooling urine and plasma from healthy subjects and carcinoid patients for diagnostic procedures available at our laboratory. Urine samples were stored at –20°C until analysis, platelet-poor plasma samples at -80°C.

Fifty µL of urine (acidified with HCl to pH 4 and containing the conservatives ascorbic acid and EDTA (1:1 w/w), added prior to collection) or 500 µL of plasma was mixed directly in an autosampler vial with 100 µL internal standard solution (final concentration 1800 nmol/L for urine and 80 nmol/L for plasma). Urine samples were diluted with 0.2 M phosphate buffer (pH 7) in ascorbic acid solution and plasma samples with ascorbic acid solution to reach a final volume of 1 mL. Fifty µL (urine sample) or 100 µL (plasma) was injected into the XLC-MS/MS system. This injection volume was equivalent to 2.5 µL of urine and 50 µL plasma.

ON-LINE SPE

Sample clean-up took place by on-line SPE, following a similar procedure as described previously for the structure related metanephrines[19]. Oasis® WCX (weak cation exchange) 10 by 1 mm SPE cartridges (Waters Corp.) were used for sample extraction. The Symbiosis® system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted by solvents delivered by two high pressure dispensers (HPD1 and HPD2). Serotonin was eluted directly on the analytical column by LC gradient elution (600 µL of mobile phase). Processing of subsequent samples was carried out in parallel and cartridges were regenerated.

LIQUID CHROMATOGRAPHY

Chromatographic separation was achieved by using an Atlantis HILIC Silica column (particle size 3 µm, 2.1 mm internal diameter by 50 mm; Waters). A gradient flow of 100 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid (A) and

acetonitrile (B) (flow rate 0.40 mL/min) was applied to the chromatographic column (Table 1). Gradients applied were linear. Column temperature was kept at 25 °C.

Mass spectrometry

The mass spectrometer was directly coupled to the chromatographic column. In positive electrospray ionization mode serotonin and its deuterated internal standard were protonated to produce ions at the form $[M+H]^+$, with *m/z* 177 and *m/z* 181, respectively. Upon collision-induced dissociation (CID) with Argon gas, these precursor ions produced characteristic product ions of *m/z* 160 [M-NH_2] and 132 [M-$C_2H_4NH_2$] and 115 [M-C2H4NH_2OH] for serotonin and *m/z* 164, 136 and 119 for the deuterated internal standard. A multiple reaction monitoring mode (MRM) was developed for the specific *m/z* transitions 177→160, 177→132 and 177→ 115 (serotonin) and 181→164, 181→136 and 181→119 (internal standard) using a dwell time of 0.05 s and an interchannel delay of 20 ms. The second and third mass transitions were used as qualifiers, following EU directive 2002/657/EC.

Statistics

For method comparison of the urinary serotonin Deming regression analysis was applied. Student's T test was applied for comparison of mean platelet-poor plasma serotonin levels in healthy controls and carcinoid patients (SPSS version 14).

Table 1. Gradient elution scheme liquid chromatography

Time (mm:ss)	Flow (mL/min)	Solvent A%	Solvent B %
00:01	0.30	5	95
01:30	0.30	20	80
03:30	0.30	20	80
04:00	0.30	5	95
06:00	0.30	5	95

Solvent A: 100 mM Amnmoniumformate pH 3; Solvent B: Acetonitrile

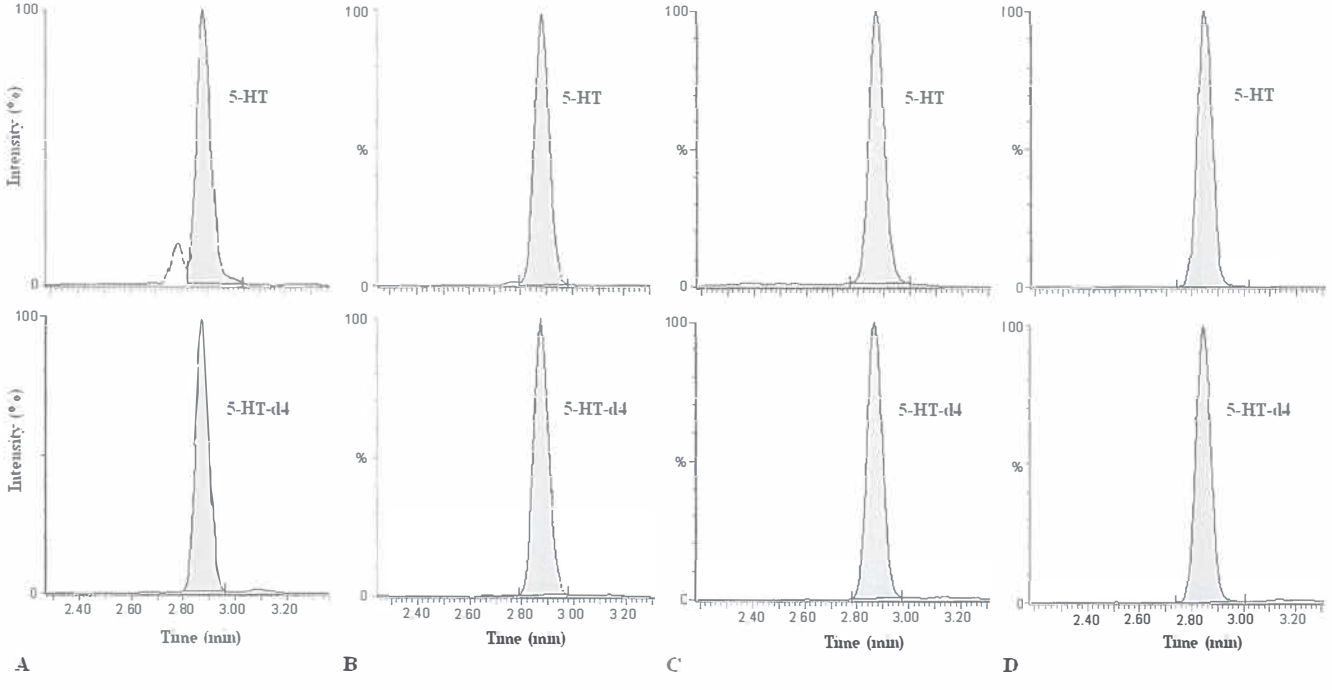

Figure 1: Chromatograms of serotonin in urine and plasma samples from healthy controls and carcinoid patients, obtained by XLC-MS/MS analysis. Chromatograms are obtained by on-line solid phase extraction using weak cation exchange coupled to hydrophilic interaction liquid chromatography with tandem mass spectoemtric detection. The mass chromatograms show the mass transitions of 177→160 (5-HT) and 181→164 (deuterated internal standard 5-HT-d4). Retention time of serotonin is 2.85 min. Responses are normalized to 100%. A: Urinary sample of a healthy control (32.4 µmol/mol creatinine), d0 area 8542, d4 area 42163. B: Urinary sample of carcinoid patient (718.5 µmol/mol creatinine), d0 area 541167, d4 area 81340. C: Plasma sample of a healthy control (4.2 nmol/L), d0 area 5181, d4 area 73091. D: Plasma sample of a carcinoid patient (289.4 nmol/L), d0 area 213186, d4 area 43549. Abbreviations: 5-HT: serotonin, 5-HT-d4: deuterated serotonin.

Table 2. Intra- and inter-assay imprecision of serotonin analysis with XLC-MS/MS

	Mean analytical variation in urine					
	Intraassay (n=20)			Interassay (n=15)		
	Mean µmol/mol creatinine	**SD** µmol/mol creatinine	**CV** %	**Mean** µmol/mol creatinine	**SD** µmol/mol creatinine	**CV** %
URINE						
Low	0.5	0.01	2.6	0.6	0.06	10.0
Medium	2.1	0.07	3.3	1.9	0.20	9.9
High	5.0	0.20	3.4	4.9	0.14	2.8
	Mean nmol/L	**SD** nmol/L	**CV** %	**Mean** nmol/L	**SD** nmol/L	**CV** %
PLASMA						
Low	5.3	0.40	7.5	5.6	0.44	7.9
Medium	174.2	2.85	1.6	165.9	8.51	5.1
High	1120.9	20.93	1.9	1083.8	44.9	4.1

Analytical variation was calculated by measuring each sample 20 times per day (intraassay) and in 20 different assays (interassay).

Results

CHROMATOGRAPHY

Total cycle time per sample, including extraction, was 6 min. Serotonin and its deuterated internal standard co-eluted after 2.85 min. The identity of the compound was confirmed by the specific mass spectrum. In Fig. 1 mass chromatograms are shown of urine and platelet-poor plasma samples from a healthy subject (A and C) and from a carcinoid patient (B and D). Ion suppression was examined by direct infusion of the analytes in the mass spectrometer with simultaneous injection of a blank sample and biological plasma and urine samples and was not observed for plasma or urine (data not shown).

DETECTION LIMITS

The limit of quantification (S/N 10:1) was 0.03 µmol/L with a CV of 5.4% (n = 20) in urine and 0.9 nmol/L with a CV of 9.0% (n = 20) in plasma.

LINEARITY AND PRECISION

For urine analysis, aqueous calibration curves in a concentration range from 0.3-7300 µmol/L (n = 8) showed excellent linearity. The mean slope was 0.4916, intercept was 0.0075 µmol/L and correlation coefficient was 0.99. Spiked urine calibration curve gave a comparable slope (0.4747), but a different intercept (0.1145 µmol/L), because of the

endogen basal serotonin concentration present in pooled urine, which confirms the selectivity of the method.

Inter-assay linearity (n=18) obtained over a concentration range from 3.5-4900 nmol/L serotonin in blank plasma was also excellent. The mean slope was 0.00165, with no significant intercept and a correlation coefficient of 0.99. An aqueous calibration curve gave a comparable slope. Intra-assay precision was determined by replicate analyses in a single run at three concentrations (n = 20). Inter-assay was determined by analysis of three concentrations over 8 weeks (n=20). For low, medium and high concentrations in both urine and plasma CVs were < 10%. Intra-assay CVs were 2.6%-3.4% in urine and 1.6%-7.5% in plasma. Inter-assay CVs were 2.8-10% in urine and 4.1%-7.9% in plasma. Precision data are shown in Table 2. Samples with high serotonin concentrations that exceed the calibration range can be diluted up to 50 times, which results in comparable outcomes.

Recovery and stability

Recovery experiments were conducted with spiked standard addition in three concentrations (low, medium, high) measured with and without solid phase extraction. Recoveries ranged from 88-135% in both urine and plasma. Absolute recovery on the SPE cartridge was measured with spiked urine samples in the same concentration ranges. These recoveries ranged from 41-74% in plasma and 83-85% in urine.

Samples, containing preservatives as described above, were found to be stable during 3 freeze-thaw cycles in both urine and plasma. Plasma samples were stable to a maximum of 2 days at 5 ºC in the dark (autosampler), and urine samples for only 8 hr. Cartridges can be reused 3 times for urine samples and up to 15 times for plasma with consistent results and without occurrence of carry-over according to the EP10-A3 protocol of the CLSI[23].

Biological samples

The new XLC-MS/MS method correlated with the former conventional HPLC method[16] with a correlation coefficient of 0.9789 (Fig. 2). The regression equation for the XLC-MS/MS method (y) and the HPLC method (x) had a slope of 1.093 (± 0.059) and an intercept of 0.081 (± 0.062) µmol. The distribution of urinary serotonin concentrations in the 120 healthy subjects was normal (Fig. 3) and the reference interval was 10-78 µmol/mol creatinine.

Free plasma serotonin concentrations were significantly higher in carcinoid patients (mean concentration 60.8 nmol/L) than in healthy controls (mean concentration 4.6 nmol/L) ($P<0.001$) (Fig 4).

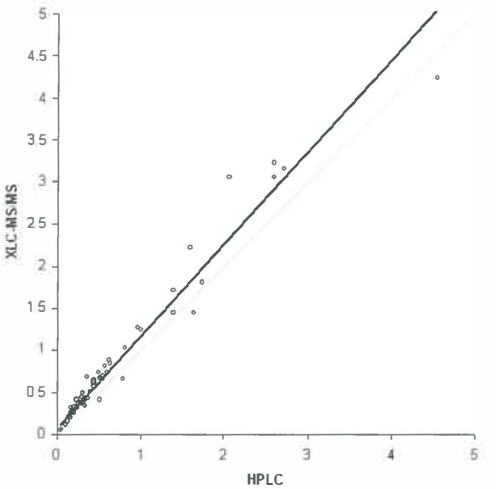

Figure 2: Correlation between the new (Y: XLC-MS/MS) and the old (X: HPLC-fluorometric detection) method for the measurement of serotonin in urine. The black line is the Deming regression scatter plot for the correlation between XLC-MS/MS and HPLC with equation y = 0.081+ 1.093 x and a correlation coefficient of 0.97. The grey line shows the ideal correlation between the two methods with the equation y =x.

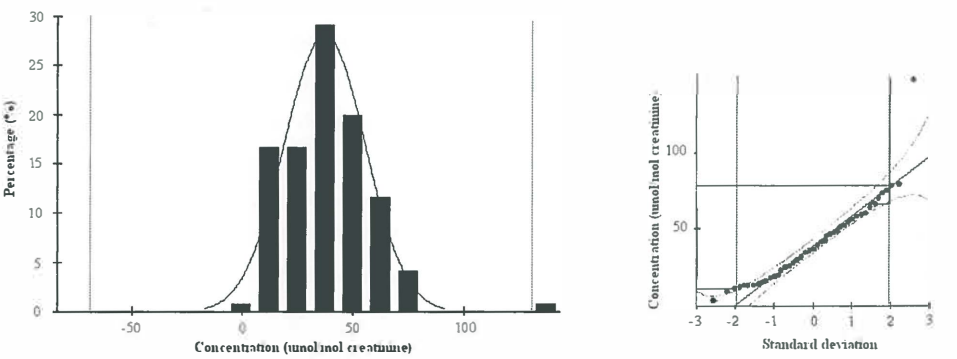

Figure 3: Distribution of urinary serotonin concentrations in reference samples from 120 healthy individuals in µmol/mol creatinine. *A*: The distribution of individual serotonin concentrations is shown in a histogram with a mean concentration of 38.2 µmol/mol creatinine. The black line shows a normal distribution which is in correspondence with the distribution of the individual serotonin concentrations. *B*: Probability plot of the distribution of individual serotonin concentrations indicating normal distribution of the concentrations.

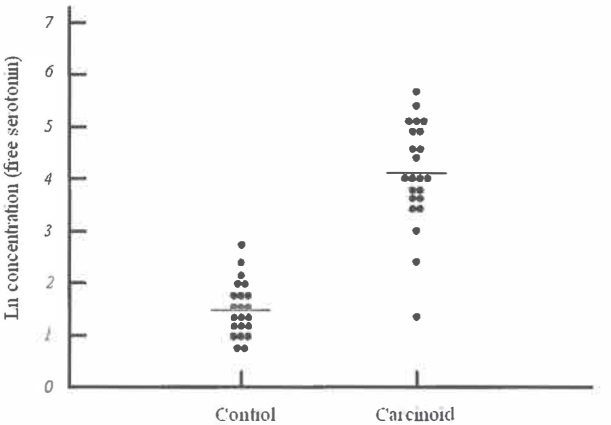

Figure 4: Free serotonin concentrations in healthy controls (n=22) and metastatic serotonin-producing carcinoid patients (n=23). Each dot represents a subject. Concentrations are expressed as the natural logarithm (ln) of nmol/L units, to obtain normal distributions. Horizontal lines indicate mean concentrations. Mean free serotonin concentrations, measured in platelet-poor plasma, are 4.6 nmol/L for healthy controls and 60.8 nmol/L for carcinoid patients. Mean serotonin concentrations in the carcinoids patients are higher than in the healthy controls ($P < 0.001$).

Discussion

This study demonstrates that plasma and urinary serotonin can be measured selective, specific and sensitive with an automated on-line solid-phase extraction-liquid chromatographic method with tandem mass spectrometric detection (XLC-MS/MS). The urinary reference interval was in agreement with previous results[11,26].

The method is based on similar analytical principles as our previously described method for the analysis of the structurally related plasma metanephrines[19]: solid-phase extraction with weak cation exchange followed by hydrophilic interaction chromatography. Major limitations of the regularly used conventional HPLC method with fluorometric detection[27] for the diagnosis of fore- and midgut carcinoid tumors include the long sample preparation time and the use of a non-isotope labeled internal standard. The newly developed automated method with MS/MS detection is, in addition, more specific because of the selection of the precursor to product mass transition, which enables identification of the analyte. Furthermore MS/MS allows the use of a stable isotope-labeled internal standard, while time for chromatographic separation can be reduced. Main advantages of the automated sample preparation are ease of handling, portability, and reduction of cost per sample, because of reduced sample preparation time, high throughput, cheaper cartridges, and reuse of cartridges. Furthermore intra- and inter-assay variation are reduced, which

increases the reproducibility of results. LC-MS/MS methods without automated sample preparation[28,29] have sensitivities and specificities that can be compared with the XLC-MS/MS method described here and are increased when compared to conventional HPLC techniques. The supplementary advantage compared to other LC-MS/MS methods is that our method is completely automated, which means a great reduction in total analysis time including sample clean-up. Specific sample preparation chemistry with weak cation exchange for clean-up and chromatographic separation with HILIC result in optimal analysis conditions.

In addition, this study demonstrated that the low physiological serotonin levels in platelet-poor plasma can be routinely measured with a more specific and sensitive method than before[26,30]. LLOQ for plasma serotonin has been reduced 70 times at our laboratory, enabling measurement of free serotonin in healthy persons. This resulted in a comparison between carcinoid patients and healthy controls with the outcome that in carcinoid patients free serotonin was 13-fold higher ($P < 0.001$) than in healthy controls. High concentrations can be hazardous in the body and result in heart valve damage and vasoactivity-related symptoms as diarrhea, flushing, and bronchoconstriction[5]. Contamination of the low platelet-poor concentrations with the high platelet-stored amount of serotonin has to be avoided. Therefore, pre-analytical factors are of importance for the measurement of serotonin in platelet-poor plasma plasma[5,25] and serotonin levels in this specific blood fraction are not consistent for healthy subjects. Our results for healthy controls are in agreement with those determined with a similar blood sampling and sample pretreatment method[25], although less careful collection and centrifugation[26] result in higher concentrations. Carcinoid patients have shown elevated concentrations previously, as in our results[26].

In order to investigate the recently uncovered role of serotonin as a peripheral hormone in processes such as liver cell regeneration[4], valvular heart disease[31] and bone formation in laboratory animals[8,32], it will be important to clarify the role of factors influencing the free serotonin fraction. The serotonin transporter, which is responsible for inward transport of free serotonin, shows considerable genetic variation, causing different levels of expression. Commonly prescribed medication such as selective serotonin reuptake inhibitors (SSRIs), influence serotonin availability by interfering with its metabolic clearance and uptake as a consequence of the blocking of the serotonin transporter[6,33]. The role of serotonin in the development of osteoporosis and bone formation has been associated with the use of those SSRIs[8,32]. During SSRI use and in patients with osteoporosis free serotonin levels are currently unknown, but should be measured in the future to investigate its role in the development and the treatment of osteoporosis.

Conclusion

Automated on-line XLC-MS/MS enables the quantification of serotonin in both urine and plasma samples. This method overcomes the limitations of existing manual HPLC procedures, especially with regard to interference and hands on analysis time. Due to the high sensitivity and specificity, low free serotonin levels in platelet-poor plasma can be measured routinely, enabling to investigate its role in serotonin-related disorders.

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Chapter 8*B*

Correspondence to

Serotonin Rising: the Bone, Brain, Bowel connection

Wilhelmina HA de Jong
Elisabeth GE de Vries
Ido P Kema

New England Journal of Medicine;2009:360(24):2580-2581.
Correction to Rosen CJ. Serotonin: the bone, brain, bowel connection;
New England Journal of Medicine;2009:360(10):957-95.

To the Editor:

Do carcinoids shed light on serotonin-induced osteoporosis? Yadav et al.[1] discovered that duodenal-derived circulating serotonin (whether in platelets or free is unspecified) inhibits bone formation. In his Perspective article, Rosen interprets this finding as an explanation for SSRI-induced osteoporosis. SSRIs significantly reduce circulating serotonin by inhibiting platelet uptake of serotonin as a consequence of blocking the serotonin transporter. Normally, circulating serotonin is almost entirely confined to platelets. We do not know the levels of free serotonin in patients taking SSRIs, but they would be expected to be elevated.

We detected highly elevated levels of serotonin in platelets and free plasma in patients with serotonin-producing metastatic carcinoid tumors, with a median level of free serotonin of 82.1 nmol/L (as compared with 4.0 nmol/L among healthy control subjects) and a median level of platelet serotonin of 18.0 nmol per $1x10^9$ platelets, as compared with 3.4 nmol per $1x10^9$ platelets among control subjects[2,3]. However, there are no obvious leads pointing to osteoporosis in such patients. Even in cases of bone metastases, we observed no changes in patients' bone-metabolism markers[4].

This discrepancy may well be due to the fact that apart from circulating serotonin, metabolic clearance plays a role. Since SSRIs also reduce serotonin clearance in peripheral transporter–expressing target organs, such as bone, serotonin-receptor activation is increased. In contrast, in patients with carcinoid tumors, transporter function is intact, and metabolic clearance can be highly up-regulated[4].

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Chapter 9

Plasma Tryptophan, Kynurenine and 3-Hydroxykynurenine Measurement Using Automated On-Line Solid-Phase Extraction HPLC–Tandem Mass Spectrometry

Wilhelmina HA de Jong
Reinier Smit
Stephan JL Bakker
Elisabeth GE de Vries
Ido P Kema

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Abstract

Tryptophan metabolism plays a key role in several (patho)physiological conditions. In order to study the clinical importance of tryptophan and its predominant metabolites (kynurenines), it is important to be able to measure large series of samples with high accuracy and reliability. We aimed to develop a high-throughput on-line solid-phase extraction-liquid chromatographic–tandem mass spectrometric (XLC–MS/MS) method that enables the measurement of tryptophan and its metabolites kynurenine and 3-hydroxykynurenine in plasma. Fifty microliters plasma equivalent was pre-purified by automated on-line solid-phase extraction, using strong cation exchange (PRS, propylsulphonic acid) cartridges. Chromatographic separation of the analytes and deuterated analogues occurred by C18 reversed phase chromatography. Mass spectrometric detection was performed in the multiple reaction-monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionization. Total run-time including sample clean-up was 8 min. Intra- and inter-assay analytical variations were less than 9%. Linearity in the 0.11–1200 (tryptophan) and 0.050 and 0.023–45 μmol/L (kynurenine and 3-hydroxykynurenine, respectively) calibration range was excellent ($R > 0.99$). Detection limits were 30 nmol/L for tryptophan, 1 nmol/L for kynurenine and 5 nmol/L for 3-hydroxykynurenine. Reference intervals for 120 healthy adults were 45.5–83.1 μmol/L (tryptophan), 1.14–3.02 μmol/L (kynurenine), <0.13 μmol/L (3-hydroxykynurenine) and 19.0–49.8 for tryptophan-to-kynurenine ratio. Blood sampling for tryptophan and tryptophan-to-kynurenine ratio should be performed before breakfast, due to biological variation during the day. This study describes how plasma tryptophan, kynurenine and 3-hydroxykynurenine can be measured accurately and precisely by automated high-throughput XLC–MS/MS.

Introduction

The essential amino acid l-tryptophan is in the body for the greater part used for protein synthesis. Tryptophan metabolism highly regulates levels of important biogenic amines such as serotonin, kynurenines, melatonin and trace amines. Tryptophan is predominantly metabolized towards neuroactive kynurenines following the kynurenine pathway. Alternative tryptophan pathways are the conversion to serotonin and melatonin, or to tryptamine and kynuramines[1,2]. A simplified scheme of the tryptophan metabolism is shown in Fig.1.

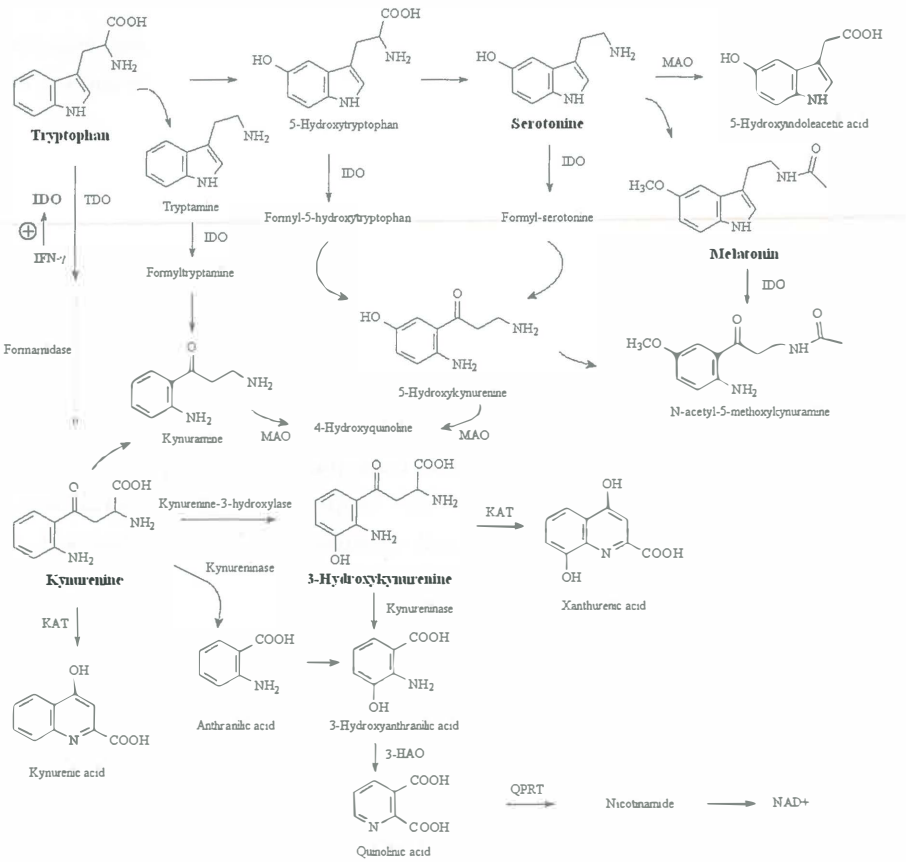

Figure 1. Schematic view of tryptophan metabolism. Abbreviations: IDO: indoleamine-2,3-dioxygenase; TDO: tryptophan-2,3-dioxygenase; IFN-γ: interferon-gamma; MAO: monoamine oxidase; KAT: kynurenine amino-transferase; QPRT: quinolinic acid phosphoribosyltransferase; NAD+: nicotinamide adenine dinucleotide.

The first metabolizing step in the kynurenine pathway is the oxidative opening of the indole ring. This reaction is catalyzed by two haem-dependent enzymes namely tryptophan 2,3-dioxygenase (TDO; EC 1.13.11.11) and indoleamine-2,3-dioxygenase (IDO; EC 1.13.11.17). TDO is almost entirely localized in the liver, while IDO is found in both the periphery (macrophages) and the central nervous system (astrocytes, infiltrating macrophages, microglia and dendritic cells). The kynurenine-to-tryptophan ratio reflects the IDO activity[3] and is therefore often used in research to monitor the tryptophan metabolism. The expression of IDO is induced during inflammation and infectious diseases by proinflammatory stimuli and T-helper-cell (T_{H}-cell) cytokines such as interferon-γ. Furthermore IDO appears to play a role in immunoregulation and communication between the immune and nervous systems[1,2,4-6] and is suggested to mediate the tumor immune escape[7]. Another kynurenine pathway metabolite, 3-hydroxykynurenine, causes neuronal damage and cell death mediated by free radicals in a neuronal hybrid cell line and the rat striatum[2,8]. In addition, it activates T_{H}-cells in immune activation.

Tryptophan metabolism plays a key role in several (patho)physiological processes, such as the endocrine manifestations of neuroendocrine tumors, i.e., carcinoid tumors with excessive serotonin production[9], mood disorders such as depression with decreased serotonin production[10], inflammation, immune-activation, transplantation[1,2,4-6] and pregnancy[10]. The metabolic fate of tryptophan (Fig. 1) is dependent on factors such as tryptophan availability and enzyme activities, and determines the synthesis of tryptophan-derived indoles, i.e., serotonin and melatonin. In order to study the (patho)physiological mechanisms of this metabolism it is important that tryptophan and its major degradative metabolite kynurenine can be easily quantified in small sample volumes with high accuracy and reliability. Several methods for detection and quantification of tryptophan and its metabolites have been described using high performance liquid chromatography (HPLC) with different detectors[3,11-16], including mass spectrometry (MS)[5,17,18]. Most of the published HPLC methods are time consuming and require large sample volumes, because separate methods are required to measure all components. HPLC coupled with electrospray ionization and tandem MS (MS/MS) is a specific and sensitive method for the detection of many endogenous compounds in biological matrices[19]. Capillary LC–MS/MS has been described for multicomponent analyses[5,18]. However, potentially considerable improvements can be made in sample throughput and accuracy by reducing the total analysis time by automation of the extraction process and coupling it directly to the chromatographic system. This process is termed on-line solid-phase extraction-liquid chromatography (XLC).

This study describes a high-throughput, sensitive, specific, and automated XLC–MS/MS method that enables simultaneous extraction, concentration, separation, and mass selective detection of tryptophan and its metabolites kynurenine and 3-hydroxykynurenine in plasma. The tryptophan-to-kynurenine ratio in plasma has been used as a diagnostic tool in several (patho)physiological conditions, i.e., leukemia[20], irritable bowel syndrome[21] and prostate cancer[22], without taking its biological variation into account. Since tryptophan plasma levels follow a circadian rhythm[23], this study also includes the measurement of the biological intra- and inter-day variation of tryptophan, kynurenine and their ratio.

Materials and Methods

Reagents

HPLC-grade acetonitrile and methanol were obtained from Rathburn Chemicals Ltd.; ammonium formate 99.995+% from Sigma–Aldrich Ltd.; formic acid 98–100% ultrapure from BDH Laboratory Supplies; isopropanol, ammonium acetate, hydrochloric acid and glacial acetic acid from Merck KGaA; and ammoniumhydroxide from ICN Biomedicals BV. Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure.

L-tryptophan, *D,L*-kynurenine and *D,L*-3-hydroxykynurenine were purchased from Sigma–Aldrich Ltd. The deuterated internal standard *L*-tryptophan-2,4,5,6,7-d_5 was from C/D/N Isotopes and *L*-kynurenine-3,4,5,6-d_4 was purchased from Buchem BV. 3-Hydroxykynurenine-d_2 was homemade[24].

Stock solutions and samples

We prepared stock solutions in 0.08 mol/L acetic acid. Stock solutions were serially diluted and used to form calibrators and low, medium, and high quality-control samples in pooled plasma via enrichment. Blank plasma was obtained by dialysis, based on a previously described method[25] of pooled plasma from healthy volunteers.

Plasma samples were obtained by venipuncture in 10 mL vacutainer tubes (Becton Dickinson) containing K_2EDTA solution as anticoagulant. After centrifugation, plasma was transferred to glass tubes, and samples were stored at −20 °C until analysis.

Before analysis, we mixed aliquots of plasma samples (250 µL) with 50 µL internal standard working solution (300 µmol/L in diluted acetic acid for tryptophan and 5 µmol/L for kynurenine and 3-hydroxykynurenine) and diluted them with 200 µL water. We placed sample vials in the autosampler, and 50 µL of each sample (equivalent to 25 µL of plasma) was injected. Required sample volume for automatic injection can be scaled down to 50 µL by using µL pickup injection mode.

INSTRUMENTATION

We used a Spark Holland Symbiosis® on-line SPE system for all analyses. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger or ACE), a solvent delivery unit (2 high-pressure dispensers), and an HPLC pump, as explained previously[26,27]. The ACE module contains 2 connectable 6-way valves and an SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and clean-up. The integrated HPLC pump was a binary high-pressure gradient pump.

We used Isolute® PRS (propylsulphonic acid based strong cation exchange) 10 mm × 1 mm SPE cartridges (Argonaut) for sample extraction and performed HPLC by use of an Atlantis dC18 column (particle size 3 µm, 2.1 mm internal diameter by 100 mm; Waters). Column temperature was controlled at 25 °C with a Mistral Column Oven (Spark Holland). Detection was performed with a Quattro® Premier tandem mass spectrometer equipped with a Z Spray® ion source operated in positive electrospray ionization mode (Waters). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

On-line SPE

We performed on-line SPE following a similar method as described before[26,27]. The Symbiosis system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted. The analytes were eluted directly on the analytical column. After conditioning, the sample was passed on to the extraction cartridge using 0.01 mol/L HCl as the loading solvent, and wash solvents were applied. Elution was performed by 300 µL 50 mM ammoniumformate pH 3, delivered by one of the high-pressure dispensers. The eluate is directly mixed with the chromatographic mobile phase and transported to the analytical column for chromatographic separation. Simultaneously, the cartridge was flushed and prepared for reuse. Processing of subsequent plasma samples was carried out in parallel.

Liquid chromatography

The binary gradient system consisted of 0.2% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Gradient elution was performed according to the elution program as shown in Table 1. Gradients applied were linear; flow rate was 0.30 mL/min. During SPE elution with a flow rate of 0.25 mL/min, the chromatographic flow rate was

decreased to 0.05 mL/min to maintain a similar total flow rate of 0.30 mL/min. Column temperature was kept at 25 °C.

Mass spectrometry

In positive ionization mode, tryptophan, kynurenine and 3-hydroxykynurenine (and their respective deuterated internal standards) were protonated to produce ions at the form $[M + H]^+$: tryptophan: *m/z* 205, tryptophan-d_5: *m/z* 210; kynurenine: *m/z* 209, kynurenine-d_4: 213, 3-hydroxykynurenine: *m/z* 225 and 3-hydroxykynurenine-d_2: 227. Upon collision-induced dissociation (CID), these precursor ions produced characteristic product ions of *m/z* 188, *m/z* 192, *m/z* 94, *m/z* 98, *m/z* 110 and *m/z* 111, respectively. We developed a multiple reaction monitoring (MRM) method using a dwell time of 10 ms and an interchannel delay of 5 ms. Recently, the use of additional MRM transitions for absolute confirmation of the presence of a compound in an analytical method has been proposed (e.g., EU directive 2002/657/EC). For this reason, we used mass transitions *m/z* 205→146 and 205→118 (tryptophan), 209→192 and 209→146 (kynurenine), and 225→208 and 225→162 (3-hydroxykynurenine) as qualifiers.

Table 1. Gradient scheme liquid chromatography.

Time (mm:ss)	Flow (mL/min)	Solvent A %	Solvent B %
00:01	0.30	100	0
00:02	0.05	100	0
01:12	0.05	100	0
01:13	0.30	100	0
01:30	0.30	100	0
04:00	0.30	60	40
06:00	0.30	60	40
06:30	0.30	100	0
08:00	0.30	100	0

Solvent A: 0.2% Formic Acid; Solvent B: Acetonitrile

QUALITY CONTROL AND METHOD VALIDATION

Selectivity

We verified the identities of sample tryptophan, kynurenine, and 3-hydroxykynurenine peaks by analysis of the compound specific mass spectra after addition of calibrator (standard addition).

Detection limits

For plasma, we determined detection limits [limit of detection (LLOD)] and quantification limits [limit of quantification (LLOQ)] by injecting serially diluted samples containing tryptophan, kynurenine and 3-hydroxykynurenine. LLOD was defined as the injected amount that produced a signal-to-noise ratio of 3 and LLOQ as the injected amount that produced a signal-to-noise ratio of 10. We estimated the percentage of carryover between sequential analyses performed on new SPE cartridges by alternating injections of blanks and plasma samples with high concentrations of the compounds.

Linearity and imprecision

We plotted the ratios of analyte peak area to internal standard peak area against metanephrines at the following 8 concentrations: 4.85, 9.70, 19.39, 38.78, 77.57, 290.88 and 1163.54 μmol/L for tryptophan, 0.19, 0.38, 0.76, 1.52, 3.04, 11.41 and 45.65 μmol/L for kynurenine, and 0.18, 0.35, 0.71, 1.41, 2.83, 10.60 and 42.39 μmol/L for 3-hydroxykynurenine. On 20 different days, we prepared and measured fresh calibration lines. The lines were calculated by use of QuanLynx software and least-squares linear regression. We applied the Clinical and Laboratory Standards Institute (CLSI) EP-6P protocol[28] to test the linearity of the method. The dilutional linearity of the assay was performed in duplicate by serial dilution of enriched plasma samples with water.

We determined intra- and inter-assay variation by the use of 3 pooled samples with tryptophan, kynurenine and 3-hydroxykynurenine in low, medium, and high concentrations and obtained intra-assay imprecision from 20 replicates measured in a single series and inter-assay imprecision from 20 different assays over a 3-week period.

Recovery

We estimated mean relative recoveries by the addition of tryptophan, kynurenine and 3-hydroxykynurenine to plasma in low, medium, and high concentrations and measured recoveries in 8 replicates of these samples by using 2 cartridges placed in series. Furthermore XLC versus LC recovery was measured using aqueous standards.

Stability

Samples with low, medium, and high concentrations of added tryptophan, kynurenine and 3-hydroxykynurenine were measured in triplicate after different storage conditions. The first set was assayed immediately and served as reference point; other sets were stored at 10 °C (autosampler temperature), 4 °C and room temperature for 16, 24, 48 and 72 h and 7 days. The remaining samples were frozen at −20 °C, and stability was investigated after 1–3 freeze–thaw cycles.

Biological variation, reference values, and patient samples

We determined biological inter-day variation by analyzing plasma obtained from 16 healthy individuals (12 men, 6 women, age range 20–56 years, median age 35 years), on 5 consecutive days (at 09:00 h). We determined biological intra-day variation at 5 times during 1 day (08:30 (before breakfast), 10:30, 12:30, 14:30, and 16:30 h) using plasma from 26 healthy individuals (13 men, 13 women, age range 21–59 years, median age 38 years) following a serotonin-low standardized diet. Calculations of the mean plasma concentrations and their 95% confidence intervals (non-parametric) were performed according to standard procedures. The plasma sample collected before breakfast (08:30 h) served as baseline concentration. Paired Student's *t*-tests were used to determine the significance of longitudinal changes in absolute analyte concentrations, with respect to the baseline concentration as well as the preceding concentration. One-sided *t*-tests were considered significant at $P<0.05$. Statistics were calculated using SPSS version 14.

Tryptophan, kynurenine, 3-hydroxykynurenine and tryptophan-to-kynurenine ratio reference intervals were based on the analysis of 120 plasma samples derived from healthy individuals (36 men, 84 women, age range 38–83 years, median age 55), during the PREVEND study[29,30]. These studies were approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent. We calculated reference intervals by use of EP evaluator[31].

Results

Quality control and method validation

Chromatography and selectivity

Total sample analysis time, including extraction, was 8 min. Complete chromatographic separation can be achieved by reversed phase chromatography. Deuterated internal standards can be used, since the mass spectrometer monitors precursor as well as product ions with high analytical specificity. Fig. 2 shows mass chromatograms of a plasma sample

derived from a healthy subject as total ion current (A) of all MRM transitions used and as the separate MRM transitions for 3-hydroxykynurenine (B), kynurenine (C), tryptophan (D) and their respective internal standards obtained by XLC–MS/MS in MRM. We confirmed the identities of the compounds by standard addition of the plasma sample, resulting in the total ion current chromatogram in Fig. 2E and the specific mass spectra in Fig. 2F–H.

Detection limits

LLOD was 30 nmol/L for tryptophan, 1 nmol/L for kynurenine, and 5 nmol/L for 3-hydroxykynurenine. Respective quantification limits (at a signal-to-noise ratio of 10) were 110, 50, and 23 nmol/L, with CVs of 13.4, 18.8, and 19.7%, respectively.

Cartridges could be reused up to 15 times, with carryover <0.1% observed between sequential analyses performed on reused SPE cartridges, by applying additional washing steps in the method.

Linearity and imprecision

Plasma calibration curves and control samples were run with every batch of patient samples. Linearity was excellent over the respective calibration ranges, with corresponding correlation coefficients (R^2) consistently >0.99 for all 3 compounds. Plasma calibration curves were reproducible between days, with $R^2 > 0.99$. Mean analytical intra- and inter-assay repeatability and reproducibility for enriched pooled plasma in low, medium, and high concentrations are shown in Table 2. Intra-assay CV (n=20) was 1.7–3.6% (tryptophan), 3.8–7.2% (kynurenine), and 4.3–8.8% (3-hydroxykynurenine). Inter-assay CV (n=20) was 1.7–7.0% (tryptophan), 2.9–5.6% (kynurenine), and 5.3–8.2% (3-hydroxykynurenine). Plasma samples with high tryptophan, kynurenine and/or 3-hydroxykynurenine concentrations that exceed the calibration range could be diluted up to 100 times.

Recovery

Recoveries ranged from 3.3 to 4.1% (tryptophan), 37.8 to 48.7% (kynurenine), and 30.9 to 47.6% (3-hydroxykynurenine).

Stability

Tryptophan and kynurenine were stable in plasma stored up to 7 days at 10, 4 °C or room temperature. 3-hydroxykynurenine is stable in plasma up to 3 days at the same conditions. No changes in measured concentrations were observed in plasma that had been subjected to 1, 2, or 3 freeze–thaw cycles. Stability data (n=3) are not shown.

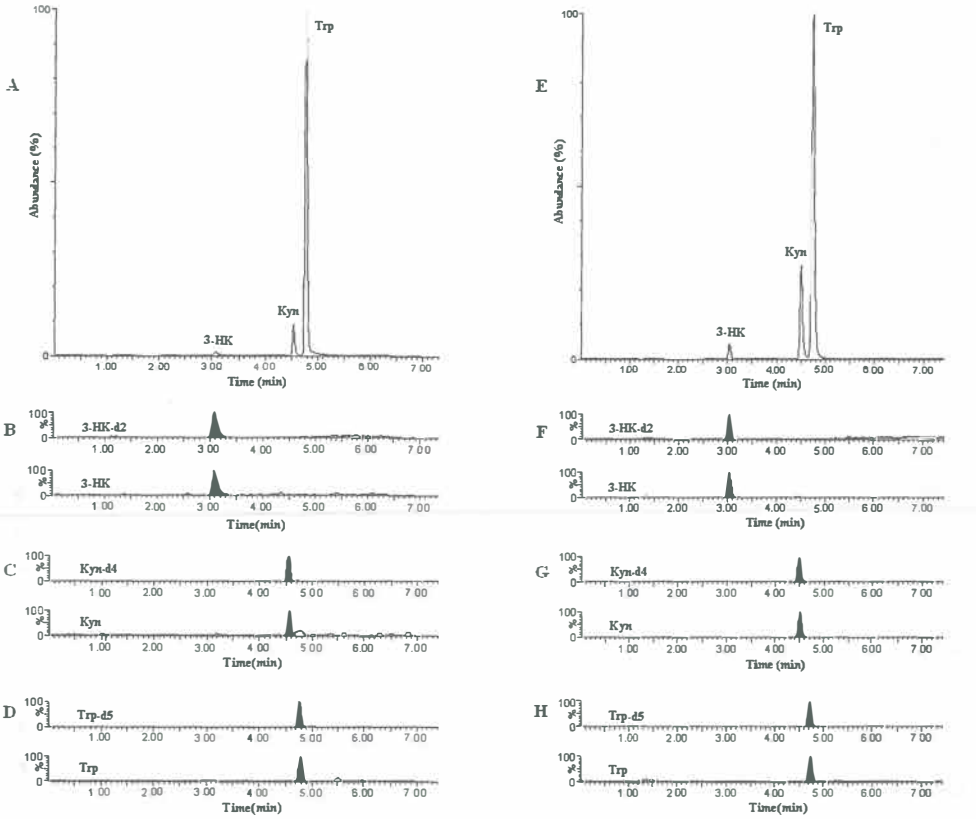

Figure 2. Total ion current mass chromatograms and mass specific chromatograms obtained in multiple reaction monitoring (MRM) mode by XLC–MS/MS for plasma tryptophan (Trp), kynurenine (Kyn), and 3-hydroxykynurenine (3-HK) and their deuterated internal standards. *A–D:* Chromatograms of plasma from a healthy subject. *E–H:* Chromatograms of plasma from a healthy subject spiked with calibrators. *A & E*: Total ion current mass chromatograms of all MRM transitions used. Retention times are 4.8, 4.5 and 3.0 min for Trp, Kyn and 3-HK, respectively. *B & F:* Mass specific chromatograms of 3-HK and its deuterated internal standard 3-HK-d_2 (*m/z* 225→110 and 227→111, respectively). *C & G:* Mass specific chromatograms of Kyn and its deuterated internal standard Kyn-d_4 (*m/z* 209→94 and 213→98, respectively). *D & H:* Mass specific chromatogram of Trp and its deuterated internal standard Trp-d_5 (*m/z* 205→188 and 210→192, respectively). All signals are normalized to full scale for the highest peak in the window. Retention time is indicated in min. The *y*-axis shows the peak abundance which is normalized to percentages relative to the highest peak in the chromatogram.

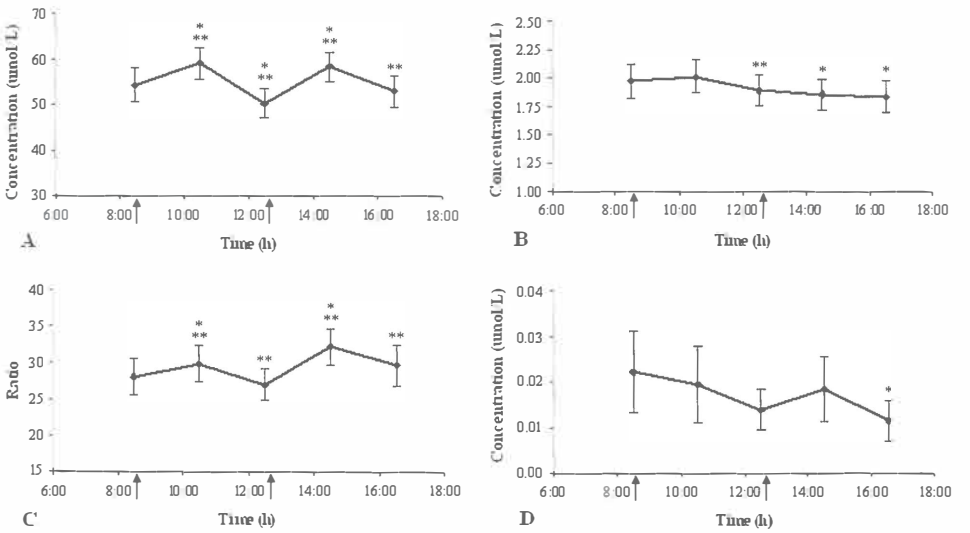

Figure 3. Biological intra-day variation of plasma tryptophan, kynurenine, tryptophan-to-kynurenine ratio and 3-hydroxykynurenine in 26 healthy subjects. A: Tryptophan. B: kynurenine. C: tryptophan-to-kynurenine ratio. D: 3-hydroxykynurenine. Plasma concentrations (µmol/L) are given as mean concentrations (n=26) with ±95% confidence interval at five times during 1 day, respectively 08:30, 10:30, 12:30, 14:30 and 16:30 h. The first time point was before breakfast. *Arrows* indicate the times bread meals were consumed. *: Significantly different (P<0.05) from baseline value (08:30 h); **: significantly different (P<0.05) from previous value.

Biological variation, reference values, and patient samples

Biological intra-day CVs (n=26) were 4.4–15.1% (tryptophan), 2.4–17.1% (kynurenine), and 33.3–116.7% (3-hydroxykynurenine), as shown in Table 2. Mean tryptophan, kynurenine, tryptophan-to-kynurenine ratio and 3-hydroxykynurenine concentrations, with 95% confidence intervals, during the day are shown in Fig. 3. This figure reveals that tryptophan concentrations are dependent on the time of day blood is sampled. After the consumption of a meal, tryptophan concentration increases significantly (P<0.05), because of the tryptophan content of food products. Kynurenine concentration decreases significantly (P<0.05) during the day (Fig. 3). Consequently the tryptophan-to-kynurenine ratio follows the same pattern as the tryptophan concentration during the day with significant changes (P<0.05). 3-Hydroxykynurenine concentration shows a not significant decrease in concentration during the day. Biological inter-day CVs (n=16) were 2.7–10.6%, 5.1–15.7% and 33.3–169.9%, respectively. Concentrations remained constant during the 5 days (data not shown).

For 120 reference samples, reference intervals were calculated with EP evaluator in a transformed parametric manner according to CLSI C28-A2[32]. Reference intervals were 45.5–83.1 µmol/L (tryptophan), 1.14–3.02 µmol/L (kynurenine), and <0.13 µmol/L (3-hydroxykynurenine). Tryptophan-to-kynurenine ratio reference values were 19.0–49.8, calculated in a parametric manner.

Discussion

Variation in tryptophan concentration due to inducible IDO/TDO degradation influences the formation of important metabolites such as serotonin. Measurement of tryptophan and its kynurenine metabolites was shown to be relevant in many pathophysiological conditions, i.e., carcinoid tumors with excessive serotonin production[9], mood disorders such as depression with decreased serotonin production[10], inflammation, immune-activation, transplantation[1,2,4-6] and pregnancy[10]. This study shows that tryptophan, kynurenine and 3-hydroxykynurenine can be measured accurately and precisely and with high-throughput using on-line SPE coupled to HPLC with MS/MS detection. In addition, the study shows that for using tryptophan and the tryptophan-to-kynurenine ratio in diagnostics or research, blood sampling has to take place before breakfast, since both markers have significant biological variation during the day. For tryptophan concentrations in plasma this circadian rhythm has been shown before[23], which is probably caused by the tryptophan content of food. As far as we know, the finding that kynurenine concentrations decrease during the day is new and should be subjected to future research on tryptophan, kynurenine and its ratio.

Measurement of plasma tryptophan and kynurenine has been executed earlier, especially with LC. The main problems for this analysis are the amphoteric characteristics of tryptophan and the concentration range difference between tryptophan and kynurenine and especially 3-hydroxykynurenine. Previously described methods, including HPLC with electrochemical, UV and fluorometric detection[3,12,16], have certain drawbacks such as labor intensity and long analysis times due to manual sample preparation. Recently, analysis times have been reduced[11] and more specific and sensitive tandem mass spectrometric detection methods have been developed[5,17,18] with reduced manual sample preparation steps. However, for the simultaneous measurement of tryptophan, kynurenine and 3-hydroxykynurenine no fast and sensitive detection method was available[15]. Since 3-hydroxykynurenine seems a potential interesting marker for the clinic and has the same characteristics as kynurenine, this component was added to our analysis.

Table 2. Intra- and interassay imprecision of the XLC-MS/MS method for plasma tryptophan (TRP), kynurenine (KYN), and 3-hydroxykynurenine (3HK).

	Mean analytical variation (n=20)						**Mean biological variation**					
	Intra-assay			Inter-assay			Inter-day (n=16)			Intra-day (n=26)		
	Mean µmol/L	**SD** µmol/L	**CV** %	**Mean** µmol/L	**SD** µmol/L	**CV** %	**Mean** µmol/L	**SD** µmol/L	**CV** %	**Mean** µmol/L	**SD** µmol/L	**CV** %
TRP												
Low	4.91	0.18	3.6	4.92	0.34	7.0						
Med	58.52	0.98	1.7	56.98	1.00	1.8	64.86	10.54	16.3	54.99	8.21	14.9
High	987.6	19.5	2.0	957.3	16.6	1.7						
KYN												
Low	0.19	0.01	7.2	0.20	0.01	5.6						
Med	2.25	0.11	4.8	2.16	0.06	2.9	1.87	0.38	20.2	1.91	0.33	17.3
High	40.43	1.55	3.8	37.65	1.78	4.7						
3HK												
Low	0.18	0.02	8.5	0.19	0.02	8.2						
Med	0.74	0.06	8.8	0.77	0.04	5.3	0.04	0.04	99.3	0.02	0.01	85.5
High	29.63	1.28	4.3	32.22	1.83	5.7						

Analytical variation was calculated by measuring each sample 20 times per day (intra-assay) and in 20 different assays (inter-assay). Intra- and inter-day mean biological variation was calculated from results from healthy individuals at 5 times during a day (0830, 1030, 1230, 1430, and 1630 h) and on 5 consecutive days at 0900 h.

Main advantages of XLC–MS/MS are ease of handling, portability, and reduction of cost per sample, because of reduced sample preparation time, high-throughput, cheaper cartridges, and reuse of cartridges[26,27]. In addition, automated sample preparation reduces analysis time and analytical variation caused by differences in manual sample pretreatment.

Because tryptophan, kynurenine and 3-hydroxykynurenine contain the same functional charged amino group, a selective SPE process can be achieved using cation exchange. Strong cation exchange media are especially suitable for these weak bases. Isolute PRS (SCX2) cartridges (Argonaut) maintain a permanent negative charge on the sorbent. PRS has very little non-polar character and is the cationic exchange sorbent (propylsulphonic acid) of choice, since the elution solvent is totally aqueous. Analytes are eluted by increasing the ion strength. For complete separation of the three components, reversed phase chromatography is used with care, since the gradient used 100% water phase, which can damage the column. Furthermore, desolvation is more difficult than with an organic solvent. However, for this purpose MS/MS detection provided enough sensitivity. Unique precursor–product ions are used for qualification and quantification. Interference with the same MRM transitions is eliminated chromatographically, whereas additional qualifiers enhance specificity of the detection method.

The XLC–MS/MS method shows excellent analytical performance. Recoveries are consistent, although below 5% for tryptophan, since the method has been optimized for kynurenine and 3-hydroxykynurenine analysis in the same run. However, tryptophan concentrations are much higher than that of kynurenine and 3-hydroxykynurenine, which means the peak abundance is still higher than that of kynurenine and 3-hydroxykynurenine. In addition, LLOQ of tryptophan is less low, although limits of quantification are still better in comparison with non-mass spectrometric methods.

The method allows reproducible quantification of plasma tryptophan, kynurenine and 3-hydroxykynurenine. Analytical variation is <9%, owing to automation of sample preparation. In addition, the required plasma volume can be scaled down to 50 µL, which enables measurement of samples from infants and neonates, mice, tissues, etc. 3-Hydroxykynurenine concentrations can still be below the quantification limit in healthy individuals.

Reference intervals in healthy subjects with XLC–MS/MS are in accordance with the ranges previously described[3,9,15,33,34].

In conclusion, plasma tryptophan, kynurenine and 3-hydroxykynurenine can be measured accurately and precisely and are reproducible by XLC–MS/MS. Samples should be collected before breakfast, since tryptophan concentration and tryptophan-to-kynurenine ratio are increased after meals. The clinical use of the method is broad, although the value of these analyses for specific diseases has to be further investigated.

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Chapter 10

Summary and Future Perspectives

Summary

The aim of this thesis was the development and clinical validation of mass spectrometric methods for the quantification of neuroendocrine biomarkers. The research was focused on quantification of biogenic amines in the clinical setting as markers for the diagnosis of neuroendocrine tumors. Neuroendocrine tumors secrete neurotransmitters and hormones, especially serotonin and catecholamines in body fluids such as blood, urine and saliva. Several methods and techniques have been developed in the last decades for the analysis of such compounds. Most of these methods are time-consuming due to extensive manual sample pretreatment and are susceptible to analytical interference because often conventional non-mass spectrometric detection techniques are applied.

In **chapter 2** an overview is given of current methods that employ (automated) liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) for the quantification of low-molecular endogenous compounds. Special attention is paid to the analysis of biogenic amines and their metabolites.

LC-MS/MS is becoming an indispensible tool in special clinical chemistry laboratories. It significantly increases analytic potential, especially in the field of low molecular weight biomarker analysis. Biogenic amines and metabolites are quantified for the diagnosis and the follow-up of patients suffering from neuroendocrine tumors. Analysis of these compounds is troublesome due to their low concentrations, instability and vulnerability for interference of other matrix constituents. Traditional techniques used for the quantification of biogenic amines and their metabolites, such as high performance liquid chromatography (HPLC) with ultraviolet, fluorometric or electrochemical detection, and to a lesser extend gas chromatographic methods and immunoassays, suffer from several drawbacks. Particular disadvantages of these techniques are labor-intensive sample preparation, long analysis times and often the relatively low specificity. Most drawbacks are circumvented by using LC-MS/MS. This technique, which has a broad analyte compatibility and high analytical performance, is rapidly evolving. In pharmaceutical and toxicological laboratories LC-MS/MS is currently the method of choice. In the last five years several LC-MS/MS assays have been implemented in special clinical chemistry laboratories to replace conventional assays of low-molecular weight biomarkers, such as steroids and biogenic amines and their metabolites.

Due to their labile nature, biogenic amines often require extensive sample preparation. Introduction of new sophisticated techniques for this purpose such as selective sorbents adsorption (e.g. immunoaffinity sorbents and restricted access media) is evolving. Developments in this area not only enable more specific analyte selection, but also

automation of the complicated clean-up procedure, when coupled to LC-MS/MS. This facilitates the handling of the growing number of samples to be analyzed in laboratories.
Thus, LC-MS/MS increases the analytic capabilities of special analyses in clinical chemistry. It overcomes drawbacks of conventional techniques and has a broad analyte compatibility and high analytical performance. Moreover it offers possibilities for the analysis of low molecular weight biomarkers such as biogenic amines and their metabolites.

Part I of the thesis deals with the analysis of catecholamines and their metabolites, specifically for the diagnosis of pheochromocytoma. Pheochromocytoma is characterized by the excessive secretion of catecholamines, resulting in symptoms such as sustained or intermittent hypertension, sweating, tachycardia, and palpitations. Highly sensitive and specific biochemical tests are required for correct clinical chemical diagnosis, avoidance of false-negative results, and follow-up of patients. Diagnosis depends on demonstration of the excessive catecholamine production, usually achieved by analysis of plasma and urinary free catecholamines and catecholamine metabolites. The 3-O-methylated metabolites, including metanephrine (MN) produced from epinephrine, normetanephrine (NMN) from norepinephrine and 3-methoxytyramine (3-MT) from dopamine, are particularly useful. Measurements of metanephrines in plasma or urine are currently recommended for the diagnosis of pheochromocytoma. Plasma free metanephrines are reported to have the highest diagnostic sensitivity and specificity for this purpose.
In **Chapter 3** we describe the development and validation of a high-throughput automated on-line solid phase extraction LC-MS/MS method (XLC-MS/MS) that enables simultaneous extraction, concentration, separation, and sensitive and mass selective detection of plasma free metanephrines for the diagnosis of pheochromocytoma. The developed XLC-MS/MS method overcomes several drawbacks of conventional methods, such as labor-intensive, offline sample preparation, long analysis times (HPLC, GC-MS), the risk of interferences (HPLC) or cross-reactivity, standardization problems and nonspecific binding (immunoassays). It uses selective SPE sample pretreatment with a weak cation exchange sorbent, reversibly interacting with the weak alkalic amine group of the metanephrine target analytes. Hydrophilic interaction chromatography for the separation of polar bases, enhances analytical sensitivity compared with traditional reversed-phase methods. This chromatographic procedure enables elution with high concentration of organic solvents, wich is more suitable for electrospray ionization. Detection is performed using a tandem quadrupole mass spectrometer operating in positive electrospray ionization mode. Due to these specific features a total analysis time, including sample clean-up, of 8 minutes is achieved. The detection ranges are in nmol/L. The XLC-MS/MS method correlates well with previously described methods regarding patient

sample results and reference intervals. Limits of quantification (0.05 nmol/L) are lowered and the minimal required plasma volume is drawn back to 50 µL This also enables measurement of samples from infants and neonates. In conclusion, Chapter 3 shows that automated XLC-MS/MS for the measurement of plasma free metanephrines is precise and linear, with short analysis time and low variable costs. The method is attractive for routine biochemical diagnosis of pheochromocytoma because of its high analytical sensitivity, the analytical power of MS/MS, and the high diagnostic accuracy of free metanephrines.

In addition to the analytical performances of the method described in Chapter 3, pre-analytical factors influencing plasma free metanephrines are examined in **Chapter 4**. Metanephrines can be affected by physiological factors (e.g. stress, posture and exercise), medications (e.g. catecholamine reuptake blockers) and diet. Especially dietary influences on plasma free metanephrines have hardly been investigated before, given the lack of well performing analysis methods. However, numerous food products, such as fruits and nuts, contain substantial quantities of biogenic amines that may produce false-positive test results. In this chapter the short-term influence of a catecholamine-rich diet on free and deconjugated metanephrines in plasma and urine is determined in 26 healthy adults. Additionally the influence of blood sampling in seated versus supine positions was examined to study previous recommendations that samples should be collected after supine rest. The subjects consumed catecholamine-rich nuts and fruits at fixed times on one day (about 35 µmol dopamine and 1 µmol norepinephrine) and catecholamine-poor products on another day. Blood and urine samples were collected at timed intervals before, during and after experimental and control interventions. Blood was obtained in supine position after 30 minutes of rest, except for the first sample which was collected in sitting position. The newly developed XLC-MS/MS method for the measurement of plasma free metanephrines described in Chapter 3 was also applied for the measurement of plasma deconjugated and urinary free metanephrines. Urinary deconjugated metanephrines were quantified using GC-MS. Chapter 4 shows that the consumption of catecholamine-rich food products has substantial effects (up to 3-fold increases) on plasma concentrations and urinary outputs of free and deconjugated 3-MT. Dietary catecholamines have negligible influences on free NMN, but substantial effects (up to 2-fold increases) on deconjugated NMN in plasma and urine. Concentrations of free and deconjugated MN in plasma and urine remain unaffected, since the parent compound epinephrine was not present in the diet. Lack of effect of the high catecholamine diet on free NMN, but the substantial effects on deconjugated NMN, reflects the importance of sulfate-conjugation for metabolism of dietary catecholamines. In addition, it confirms previous observations that there are different sources of free and sulfate-conjugated NMN and that free metanephrines have a faster circulatory clearance

than conjugated compounds with different sites of production. Thus dietary restrictions should be considered to minimize false-positive results for urinary and plasma deconjugated metanephrines during diagnosis of pheochromocytoma. Similar considerations appear warranted for plasma and urinary free 3-MT, but not for free NMN or MN, indicating advantages of measurements of the free compared to deconjugated metabolites.
Chapter 4 also describes that while the free metanephrines are relatively insensitive to dietary factors, they are quite sensitive to the conditions of blood sampling, showing rapid decreases within 30 minutes of supine rest after insertion of an intravenous catheter. The lesser effect on MN than NMN is attributable to the substantial amount of circulating MN produced within adrenal medullary cells, independently of epinephrine release. Lack of influence of supine rest on plasma deconjugated metanephrines is explained by the much slower circulatory clearance of the sulfate-conjugated than the free metabolites.

The parent catecholamines can also be measured in body fluids themselves to support identification of neuroendocrine disorders. Although analysis of catecholamines, due to their intermittent release in contrast to the continuous production of metanephrines, is less sensitive and specific for pheochromocytoma diagnostics, the measurement of catecholamines is commonly applied. Reliable techniques for catecholamine quantification are required. The currently applied methods use laborious sample preparation techniques coupled to detection techniques that are vulnerable for interferences and are technically demanding. Aim of the research described in **Chapter 5** is the development of a rapid, specific and sensitive automated XLC-MS/MS method for the quantification of catecholamines in urine. The developed XLC-MS/MS method overcomes drawbacks such as labor intensity, offline sample preparation, long analysis times and the risk of interferences. It uses selective SPE sample pretreatment with phenyl boronic acid (PBA) sorbent, resulting in reversible complex formation of the sorbent with the catechol group of catecholamines. Separation of the three catecholamines is accomplished with a pentafluorophenyl propyl reversed-phase chromatographic column. Detection is performed using a tandem quadrupole mass spectrometer operating in positive electrospray ionization mode. Due to the specific features a total analysis time, including sample clean-up, of 14 minutes is achieved. The XLC-MS/MS method correlates well with the previously used offline HPLC method with electrochemical detection with a correlation coefficient of >0.98. Reference intervals, set by analyzing 24h-urine collections of 120 healthy subjects, are 1-10, 10-50 and 60-225 µmol/mol creatinine for epinephrine, norepinephrine and dopamine, respectively. In conclusion, this method for the quantification of urinary catecholamines with XLC-MS/MS combines highly selective PBA SPE sample preparation

with specific and sensitive tandem mass spectrometric detection. Automation results in increased accuracy, greatly reduced analysis time and high throughput.

The high analytical performance of this method enables the investigation of pre-analytical influences similar to the study described in Chapter 4 regarding plasma metanephrines. Therefore in **Chapter 6** the influence of diet composition on the concentration of urinary catecholamines is investigated in 27 healthy subjects. The catecholamine-rich diet substantially increases deconjugated norepinephrine (up to 10-fold) and dopamine (up to 20-fold) while the free fraction is less, but significantly, influenced (up to 1.5-fold for free dopamine). Measurement of urinary free catecholamines should therefore prevail above measurement of deconjugated catecholamines. Repeated measurements with dietary restrictions prior to sample collection are warranted to confirm the presence of increased urinary free catecholamine excretion. Alternatively, plasma free metanephrines could be measured, which are not affected by dietary catecholamines, as described in Chapter 4.

Part II focuses on the measurement of tryptophan-related compounds (indoles). Tryptophan-derived compounds play an important role in many (patho)physiological processes. Excessive serotonin production, for example, is known to be the major cause of endocrine manifestations of carcinoid tumors. Decreased tryptophan and thus serotonin availability is implicated in several psychiatric diseases, such as depression. Decreased tryptophan availability and production of neurotoxic intermediates such as quinolinic acid are known to occur during, immune-activation, following e.g. transplantation and pregnancy. This is caused by induction of enzymatic tryptophan degradation towards the kynurenine pathway. The metabolic fate of tryptophan is dependent on factors such as tryptophan availability and enzyme activities, and determines the synthesis of tryptophan derived indoles, i.e. serotonin and melatonin.
Serotonin-producing carcinoid tumors occur most frequently in the midgut and to a lesser extent in foreguts. When metastasized, symptoms of the carcinoid syndrome, including flushing, diarrhea, right-sided hart disease and bronchoconstriction, can occur. The tumor gives rise to increased serotonin synthesis from its precursor tryptophan, thereby reducing tryptophan levels available for other metabolic routes. 5-HIAA is the final breakdown product of serotonin. The quantification of this metabolite in urine is the most commonly used test for follow-up of carcinoid patients. In **Chapter 7** we describe the development and clinical validation of an XLC-MS/MS method for the analysis of 5-HIAA in urine. The resulting method has a lower imprecision and time per analysis than manual extraction methods and higher sensitivity and specificity than non-mass spectrometric detection techniques. Automated pre-purification of urine is carried out with cartridges containing

strong hydrophobic polystyrene resin. The chromatography is also based on hydrophobic interaction. Detection is performed using a tandem quadrupole mass spectrometer operating in positive electrospray ionization mode. This procedure results in a total analysis time, including sample clean-up, of 5 minutes. Absolute recovery is 96.5%-99.6%. Analytical variation is <5%. Results of patient samples with known or suspected metastatic carcinoid tumors obtained by XLC-MS/MS highly correlate with the previously used HPLC method with fluorometric detection (R^2=0.99). Thus, in comparison with the conventional HPLC method, the XLC–MS/MS method described in Chapter 7 for the routine measurement of urinary 5-HIAA demonstrates improved precision, sensitivity and specificity with a significantly shorter time per analysis enabling high-throughput analysis.

In **Chapter 8, part A** we describe the development of an XLC-MS/MS method for the quantification of serotonin in urine. This method should replace the previously used laborious conventional liquid chromatographic technique with manual sample pretreatment and fluorometric detection. Urinary serotonin is a marker that has been implicated in the diagnosis of unusual foregut carcinoid tumors. The analytical principle is comparable to that of the metanephrine assay described in Chapter 3, in which weak cation exchange and hydrophilic interaction chromatography were used for sample clean-up and separation. Detection is performed using a tandem quadrupole mass spectrometer operating in positive electrospray ionization mode. This resulted in a method with a total run-time, including sample clean-up, of 6 minutes with excellent linearity from 30-7300 µmol/L and analytical variation <10%. Serotonin stored in platelets is the most accurate marker for the detection of small amounts of serotonin overproduction and therefore an important marker for the diagnosis of (metastatic) carcinoids. The plasma free serotonin fraction probably is the active fraction which is hazardous for the body. Chapter 8 describes how free serotonin can be measured in platelet-poor plasma by specific and sensitive XLC-MS/MS with a lower quantification limit of 0.9 nmol/L. Free plasma serotonin concentrations in metastatic midgut carcinoid patients are demonstrated to be 13-fold higher than in controls. The possible role of plasma free serotonin is thought to be important in the development of SSRI-derived osteoporosis. In **Chapter 8, part B** this hypothesis is discussed by demonstrating that in metastatic carcinoid patients plasma free serotonin levels are significantly elevated (compared to healthy controls). However, no osteoporosis is observed in these patients. An explanation for this discrepancy might be the role of metabolic clearance. Since SSRIs also reduce serotonin clearance in peripheral transporter–expressing target organs, such as bone, serotonin-receptor activation is increased. In contrast, in patients with carcinoid tumors, transporter function is intact, and metabolic clearance may be highly up-regulated.

Carcinoid patients convert most of the food-derived tryptophan to serotonin. Long-tem augmentation of the serotonin biosynthesis may result in serious reduction of the free tryptophan body pool, possibly causing deficiencies in other tryptophan metabolic pathway components, such as kynurenine, 3-hydrokynurenine and the end-product niacine. The kynurenine-to-tryptophan ratio reflects the activity of the enzyme indoleamine deoxygenase (IDO). The expression of IDO is especially induced by pro-inflammatory stimuli such as cytokines in conditions such as inflammation and infection and has further been found to play an important role in tumor immune escape. Aim of the research described in **Chapter 9** is the development of an XLC-MS/MS method for the simultaneous quantification of tryptophan, kynurenine and 3-hydroxykynurenine. It uses selective SPE sample pretreatment with strong cation exchange sorbents, reversibly interacting with the amine group of the compounds. Chromatographic separation is achieved by C18 reversed phase chromatography. Detection is performed using a tandem quadrupole mass spectrometer operating in positive electrospray ionization mode. This resulted in a total analysis time of 8 minutes, including sample clean-up. The minimal required plasma volume is drawn back to 10 μL, which enables measurement of samples from mice and rats. Analytical variation is <9%. Detection limits were 30 nmol/L for tryptophan, 1 nmol/L for kynurenine and 5 nmol/L for 3-hydroxykynurenine. Reference intervals in plasma were comparable to previously published values. In order to minimize biological variation, blood sampling for tryptophan and tryptophan-to-kynurenine ratio should preferably be performed before at a fixed timepoint in a fasting state. In conclusion, Chapter 10 shows that automated XLC-MS/MS for the measurement of plasma tryptophan, kynurenine and 3-hydroxykynurenine is precise and linear, with short analysis time and low variable costs.

Future perspectives

This thesis deals with the development and clinical chemical validation of XLC-MS/MS methods for the determination of neuroendocrine biomarkers in clinical chemistry. New analytical methods have been developed or transferred from conventional off-line HPLC methods towards automated methods with tandem mass spectrometric detection. Furthermore, studies have been performed in which the applicability of the newly developed assays was tested. Several XLC-MS/MS measurements, such as plasma free metanephrines, urinary serotonin, urinary 5-HIAA and urinary catecholamines, described in this thesis are currently used as routine methods. Nowadays the number of applications that is implemented in routine clinical chemistry increases significantly. For the future LC-

MS/MS appears to be the method of choice for low-molecular weight biomarker testing, which necessitates further effort to be put in the development of LC-MS/MS methods for the quantification of other biogenic amines and steroids.

On the one hand, future research should focus on the further development of LC-MS/MS methods since their analytical opportunities are numerous. Special attention should be paid to sample pretreatment techniques. For instance, immunoaffinity application should be investigated, since limits of conventional SPE are reached and regular SPE is often not sufficient for most steroid compounds that are pathophysiologically important in diagnosis and treatment of many endocrine disorders. An additional analytical challenge is to investigate to what extent the binding between proteins such as vitamine D binding protein and sex hormone binding globulin and target analytes (mainly steroids and hormones) can be dissolved. This is necessary to elucidate what fraction, free or total, is measured. Furthermore, ionisation efficiency can be optimized for several compounds (i.e. norepinephrine and normetanephrine) to enable a more sensitive and accurate measurement. In the near future, developments of assays for steroids deserve priority among assays for other biogenic amines and small molecular weight compounds. XLC-MS/MS is very versatile and there are numerous compounds for which it is expected that they can be measured with this technique.
Future research should furthermore exploit the opportunities given by high-throughput, specificity and sensitivity. A major advantage of XLC-MS/MS in clinical chemistry is the fact that it permits to analyze large numbers in short periods of time. Numerous data can be collected to build data bases enabling analysis in prospective and retrospective cohorts. For example, reference values for biomarkers quantified by LC-MS/MS can easily be established by measuring a high volume of samples from healthy controls. With the conventional techniques it is almost impossible to handle the large number of samples required for these purposes. Given the high specificity of this technique, usually unknown pre-analytical physiological influences of medications on test results can be examined. The high sensitivity of LC-MS/MS enables measurements in matrices with low concentration ranges, such as saliva, liquor cerebrospinalis, and tissue (dialysis) samples, while no significant changes are required in the methods developed for plasma or urine.

On the other hand, clinical application of the analysis of neuroendocrine biomarkers might give new insights for diagnosis and treatment of neuroendocrine disorders. Because of the measurable low concentrations and the availability of a broad range of biomarkers, it might be possible to differentiate between patient groups An important matter to be unravelled is the clinical value of plasma free 3-MT in patients suffering from dopamine (metabolite)

producing tumors such as neuroblastoma and paraganglioma in comparison to healthy subjects. Previously, it was impossible to measure the plasma free concentrations of 3-MT in healthy subjects due to very low concentration ranges and to the occurrence of interferences.

The in this thesis described analytical method allows suspection of the presence of a pheochromocytoma when metanephrines levels are slightly elevated, compared to reference values, and the small size of the tumor still prevents that imaging techniques can detect the tumor. However, to address this issue further research is required. Pre-analytical physiological factors can be important for the interpretation of results and are most of the time unknown. This thesis dealt with a number of such physiological factors with respect to plasma metanephrines, such as diet and blood sampling posture. However, in order to increase diagnostic specificity of the method, other factors influencing the plasma free metanephrines concentrations still need investigation. Examples are the influence of hypertension or the use of medication interfering with catecholamine pathways.. In this thesis it is demonstrated that diet restrictions are required when the deconjugated fraction of the metanephrines is measured. Therefore, diagnostic specificity of urinary free metanephrines is expected to be higher than that of the deconjugated fraction, as is the case in plasma. The diagnostic sensitivity and specificity of urinary free and deconjugated metanephrines for the diagnosis of pheochromocytoma need to be compared in a large prospective study. When such studies are performed, conclusions can be drawn about the replacement of the analysis of urinary deconjugated metanephrines by that of the urinary free fraction, which is important, since urinary deconjugated metanephrines are commonly used for diagnosis of pheochromocytoma. Several studies showed that measurement of plasma free metanephrines is the superior method for the diagnosis of pheochromocytoma. In urine however, concentrations of free metanephrines are higher than in plasma and therefore measurable with less advanced equipment. Free serotonin in plasma will be used to investigate its concentration in healthy controls, carcinoid patients and depressed patients on SSRI medication. Serotonin is the compound responsible for the symptoms of carcinoid patients. Genetic polymorphisms of the serotonin transporter in those patient groups need to be examined in order to investigate its effect on clearance of free serotonin.

The developed method for the quantification of tryptophan, kynurenine and 3-hydroxykynurenine enables the elucidation of the role of the enzyme indole-2,3-dioxygenase (IDO) in several pathophysiological conditions. Several research groups perform currently studies to clarify mechanisms in those conditions (i.e. inflammation and immune activation) by measurement of plasma, urine, liquor and cell or tissue homogenate. IDO measurements might be of interest to unravel the relation between immune activation

and mood disorders such as depression. IDO is an immunosuppressive enzyme that can also be expressed by tumor cells and is therefore suggested to be involved in the mechanism of tolerance in malignancy. Demonstrating the IDO activity in tumor tissues and combining it with the tryptophan-to-kynurenine ratio might indicate the degree of malignancy. IDO activity and impediments in tryptophan-to-kynurenine ratio under several conditions, such as inflammation, graft versus host rejection in transplantation immunology and dialysis, need further research. In addition to the measurement of kynurenine and 3-hydroxykynurenine, assays have to be developed for the measurement of other kynurenine pathway compounds, such as kynurenic acid, quinolic acid, anthranillic acid and niacine.

Nederlandse samenvatting

Het doel van het in dit proefschrift beschreven onderzoek was de ontwikkeling en klinische validatie van massaspectrometrische analysemethoden voor neuroendocriene biomarkers, in het bijzonder biogene aminen. Deze biomarkers worden gebruikt bij de biochemische diagnose van neuroendocriene tumoren. Neuroendocriene tumoren zijn zeldzame, meestal langzaam groeiende, tumoren die neurotransmitters en hormonen zoals serotonine en catecholaminen aanmaken. Voor de kwantificering van biogene aminen en hun metabolieten in lichaamsvloeistoffen zoals bloed, urine en speeksel worden meestal interferentie-gevoelige en bewerkelijke conventionele niet-massaspectrometrische methoden gebruikt. In het hier beschreven onderzoek is gebruik gemaakt van een, voor de klinische chemie, nieuwe combinatie van geautomatiseerde voorzuiveringsapparatuur met vloeistofchromatografie en tandem massaspectrometrie. De analytisch uitmuntend presterende ontwikkelde methodes zijn gebruikt om ondubbelzinnig de invloed van pre-analytische factoren, zoals dieet, op concentraties van neuroendocriene markers in plasma en urine vast te stellen.

In **hoofdstuk 2** wordt een literatuuroverzicht gegeven van de huidige (geautomatiseerde) vloeistofchromatografische technieken met tandem massaspectrometrische detectie (LC-MS/MS), die gebruikt worden voor de kwantificering van laag-moleculaire, door het lichaam zelf geproduceerde stoffen. Hierbij is de nadruk gelegd op de analyse van biogene aminen en hun afbraakproducten. LC-MS/MS is een snel groeiende techniek binnen de klinisch chemische laboratoria. Door toepassing van deze techniek nemen analytische mogelijkheden toe, vooral voor biomarkers met een laag molecuul gewicht. De tot deze groep behorende biogene aminen en hun afbraakproducten worden gekwantificeerd voor de diagnose en het vervolgen van patiënten met neuroendocriene tumoren. Lage concentraties en instabiliteit van biogene aminen en interferentie-gevoeligheid voor andere matrix componenten bemoeilijken de analyse. Traditionele technieken zoals vloeistofchromatografie (HPLC) met ultraviolette, fluorometrische of electrochemische detectie, en in mindere mate gaschromatografische methoden en immunoassays hebben verschillende tekortkomingen. Voorbeelden van tekortkomingen zijn de arbeidsintensieve monstervoorbewerking, de lange analyse tijden en de relatief lage specificiteit. Met LC-MS/MS worden de meeste tekortkomingen omzeild. Deze bepalingstechniek is breed toepasbaar voor de nauwkeurige kwantificering van verscheidene groepen verbindingen, ook in het lage detectiegebied. In de farmaceutische industrie en toxicologie zijn LC-MS/MS bepalingen tegenwoordig de eerste keus. De laatste jaren laten ook een toename zien van LC-MS/MS bepalingen in klinisch chemische laboratoria.

Voor de isolatie van biogene aminen uit lichaamsvloeistoffen zijn procedures beschreven die berusten op selectieve interactie tussen de te meten moleculen en kolom dragermaterialen. Ontwikkelingen op dit gebied leveren niet alleen selectieve isolatie van te bepalen markers op, maar maken het ook mogelijk de gehele monstervoorbewerkingsprocedure te automatiseren. Deze geautomatiseerde monsteropwerking kan direct gekoppeld worden aan de LC-MS/MS, wat resulteert in snelle en reproduceerbare verwerking van monsters. Dit is een groot voordeel bij de verwerking van het groeiend aantal te analyseren monsters.

Deel I van het proefschrift beschrijft de analyse en klinische validatie van catecholaminen en bijbehorende metabolieten, met de nadruk op de diagnose van de bijniertumor het feochromocytoom. Een feochromocytoom wordt gekarakteriseerd door de overmatige uitscheiding van catecholaminen en gaat meestal gepaard met symptomen zoals een hoge bloeddruk, zweten en een hoge hartslag. Voor de correcte klinisch chemische diagnose, het vermijden van vals-negatieve resultaten en de follow-up van patiënten, zijn hoogsensitieve en specifieke biochemische testen vereist. Deze berusten op het aantonen van de overmatige catecholamineproductie door de analyse van catecholaminen en metabolieten in plasma en urine. Vooral de 3-O-gemethyleerde metabolieten, metanefrine (MN) uit adrenaline, normetanefrine (NMN) uit noradrenaline en 3-methoxytyramine (3-MT) uit dopamine, zijn belangrijk. De analyse van metanefrinen in plasma of urine wordt in de literatuur aanbevolen als de beste test voor de diagnose van feochromocytoom, waarbij is beschreven dat de analyse van plasma metanefrinen de hoogste sensitiviteit en specificiteit heeft.

In **hoofdstuk 3** beschrijven we de ontwikkeling en klinische validatie van een high-throughput LC-MS/MS methode met geautomatiseerde monstervoorbewerking (XLC-MS/MS) voor de kwantificering van plasma vrije metanefrinen. De methode is in staat om achtereenvolgens extractie, concentratie en scheiding van metanefrinen uit te voeren waarna gevoelige massa-selectieve detectie plaatsvindt. Deze methode kent geen van de tekortkomingen van andere methodes, zoals bewerkelijke offline monstervoorbewerking, lange analysetijd (HPLC, GC-MS), risico op interferenties (HPLC) of kruis-reactiviteit, standaardisatieproblemen en niet-specifieke binding (immunoassays). De toegepaste selectieve vaste fase extractie (SPE) monstervoorbewerking berust op een ionuitwisseling van het zwak negatief geladen dragermateriaal met de positief geladen aminegroep van de metanefrinen. De chromatografische scheiding van de componenten wordt uitgevoerd door middel van hydrofiele interactie chromatografie, een principe speciaal ontwikkeld voor de scheiding van polaire basen. Hiermee wordt de analytische sensitiviteit aanzienlijk verbeterd ten opzichte van traditionele hydrofobe interactie vloeistofchromatografie, omdat

de gebruikte hoog-organische elutie de ionisatie van stoffen via positieve electrospray verbetert. Voor de detectie wordt een quadrupole tandem massaspectrometer gebruikt. De zo opgebouwde methode heeft een totale analysetijd van 8 minuten; dit is inclusief de monstervoorbewerking. De XLC-MS/MS methode correleert goed met eerder beschreven methoden zowel ten aanzien van concentraties in patiëntenmonsters als gerapporteerde referentieintervallen. Kwantificeringslimieten zijn echter lager (tot maximaal 0.05 nmol/L) en het minimaal vereiste plasmavolume is teruggebracht tot 50 µL, wat meting van monsters afkomstig van kinderen en pasgeborenen mogelijk maakt. Samenvattend wordt in hoofdstuk 3 beschreven dat de ontwikkelde XLC-MS/MS voor de analyse van plasma vrije metanefrinen precies en lineair is en een korte analysetijd en lage variabele kosten heeft. De methode is aantrekkelijk voor routine diagnostiek van het feochromocytoom vanwege de hoge analytische gevoeligheid en specificiteit van tandem massaspectrometrie en de hoge diagnostische accuraatheid.

Aanvullend op de in hoofdstuk 3 beschreven analytische karakteristieken van een plasma vrije metanefrinen bepaling, worden in **Hoofdstuk 4** pre-analytische invloeden op deze catecholamine-metabolieten uitgezocht. Metanefrine concentraties kunnen namelijk beïnvloed worden door fysiologische factoren, zoals stress, houding en inspanning, medicatie, of dieet. De invloed van dieet op plasma vrije metanefrinen is nauwelijks onderzocht, omdat de hiervoor benodigde analytische specificiteit niet werd gehaald door eerder beschreven analysemethoden. Dergelijk onderzoek is uitermate belangrijk aangezien verscheidene etenswaren, vooral fruit en noten, substantiële hoeveelheden biogene aminen bevatten die potentieel vals-positieve resultaten kunnen geven. In Hoofdstuk 4 wordt daarom het korte termijn effect van een catecholamine-rijk dieet op de vrije en gedeconjugeerde (vrije en sulfaat-geconjugeerde) metanefrinen concentratie in plasma en urine in 26 gezonde volwassenen bestudeerd. De proefpersonen consumeerden gedurende 1 dag op vaste tijden catecholamine-rijke noten en fruit (circa 35 µmol dopamine en 1 µmol noradrenaline) en op een andere dag catecholamine-arme producten. Bloed en urinemonsters werden verzameld op vaste momenten voor, tijdens en na de experimentele en controle maaltijden. Bloed werd afgenomen in liggende positie na 30 minuten rust, met uitzondering van de eerste bloedafname die in zittende positie werd afgenomen. Gedeconjugeerde metanefrinen in plasma en vrije metanefrinen in urine zijn ook gekwantificeerd met de methode beschreven in Hoofdstuk 3. Gedeconjugeerde metanefrinen in urine zijn bepaald met GC-MS. De resultaten in hoofdstuk 4 tonen aan dat het eten van catecholamine-rijk voedsel substantiële effecten heeft op vrij en gedeconjugeerd 3-MT in plasma en urine (tot drievoudige toename in concentratie). Dit terwijl in deze matrices verwaarloosbare effecten op vrij NMN, maar substantiële effecten

op gedeconjugeerd NMN (tot tweevoudige toename) gevonden worden. Vrij en gedeconjugeerd MN worden niet beïnvloed, aangezien het genuttigde voedsel de MN-voorloper adrenaline niet bevatte. De duidelijke effecten op gedeconjugeerd NMN, zonder dat de hoeveelheid vrij NMN wordt beïnvloed, laat het belang zien van sulfaat-conjugatie in het metabolisme van exogene catecholamines.. Bovendien bevestigt dit resultaat de eerdere bevindingen dat vrij en sulfaat-geconjugeerd NMN uit verschillende bronnen afkomstig zijn, dat vrije metanefrinen sneller worden geklaard dan geconjugeerde verbindingen en dat dit op verschillende locaties plaatsvindt. Dus om de kans op vals-positieve uitslagen van gedeconjugeerde metanefrinen te minimaliseren, moeten dieetrestricties voorafgaand aan monsterinzameling van zowel plasma als urine worden overwogen. Het instellen van dieetrestricties lijkt ook noodzakelijk bij de bepaling van vrij 3-MT, maar niet voor vrij NMN of MN. Het bepalen van de vrije fractie heeft daarom de voorkeur.

In hoofdstuk 4 wordt verder beschreven dat de plasma vrije metanefrinen gevoelig zijn voor de houding tijdens bloedafname. Na 30 minuten liggen is een substantiële afname van plasma vrij MN en NMN te zien in vergelijking met directe bloedafname na plaatsing van een intraveneuze naald. Dat er beduidend minder effect optreedt voor MN dan voor NMN is te verklaren doordat MN voornamelijk wordt geproduceerd in de bijniermergcellen onafhankelijk van sympathische adrenalineafgifte. Plasma gedeconjugeerde metanefrinen worden niet beïnvloed doordat sulfaat-geconjugeerde metabolieten langzamer worden geklaard dan vrije metabolieten.

Ook de catecholaminen zelf kunnen in lichaamsvloeistoffen worden bepaald voor de identificatie van neuroendocriene afwijkingen. Catecholaminen worden pulsgewijs afgegeven in tegenstelling tot metanefrinen die continue geproduceerd worden. Hoewel hierdoor de analyse van catecholaminen minder sensitief en specifiek is voor de diagnostiek van het feochromocytoom, wordt deze nog veel toegepast binnen de klinische chemie, vaak als aanvulling op de metanefrine bepaling. De huidige toegepaste methoden berusten op tijdrovende handmatige monstervoorbewerking gekoppeld aan detectie technieken die interferentie-gevoelig zijn. Het doel van het onderzoek beschreven in **Hoofdstuk 5** is de ontwikkeling van een snelle, specifieke en gevoelige geautomatiseerde XLC-MS/MS methode voor de kwantificering van catecholaminen in urine. De tekortkomingen van conventionele methodes, zoals intensieve en tijdrovende monstervoorbewerking, lange analysetijd en risico op interferenties zijn hiermee overwonnen. Selectieve monstervoorbewerking door middel van vaste fase extractie (SPE) berust op reversibele complexvorming tussen de catecholgroep van de catecholaminen en het fenylboraat dragermateriaal (PBA) op de cartridge. Chromatografische scheiding van de drie catecholaminen gebeurt op basis van hydrofobe interactie met een pentafluorofenyl propyl

kolom. Detectie vindt plaats met positieve electrosprayionisatie in een quadrupole tandem massaspectrometer. De zo opgebouwde methode heeft een totale analysetijd van 14 minuten, inclusief monstervoorbewerking. De XLC-MS/MS methode correleert goed met de voormalige offline HPLC methode met electrochemische detectie (correlatie coëfficiënt van >0.98). Referentiewaarden, bepaald door de analyse van 24h-urine verzamelingen afkomstig van 120 gezonde personen waren respectievelijk 1-10, 10-50 and 60-225 µmol/mol creatinine voor adrenaline, noradrenaline en dopamine. Samenvattend combineert de beschreven XLC-MS/MS methode voor de kwantificering van catecholaminen in urine de hoge selectiviteit van PBA SPE met de hoge specificiteit en sensitiviteit van de tandem massaspectrometrische detectie. Automatisering resulteert in een toegenomen accuraatheid,en een veel kortere opwerk- en analysetijd.

De hoge analytische kwaliteit van deze methode voor de bepaling van catecholaminen in urine maakt het mogelijk de invloed van pre-analytische fysiologische factoren op de test uitslagen te onderzoeken, zoals eerder beschreven in Hoofdstuk 4 voor plasma metanefrinen. In **Hoofdstuk 6** is de pre-analytische invloed van dieetsamenstelling op de concentraties van catecholaminen in urine onderzocht in 27 gezonde personen. Het catecholamine-rijke dieet verhoogt gedeconjugeerd noradrenaline (tot 10 keer) en dopamine (tot 20 keer) fors, terwijl de vrije fractie minder, maar ook significant stijgt (tot 1,5 keer voor vrij dopamine). De bepaling van vrije catecholaminen verdient daarom de voorkeur boven de bepaling van urine gedeconjugeerde catecholaminen. Voorafgaand aan de monsterinzameling zijn dieetrestricties noodzakelijk. Als alternatief kunnen plasma vrije metanefrines worden bepaald, aangezien die niet worden beïnvloed door catecholamines uit de voeding, zoals beschreven is in hoofdstuk 4.

In **Deel II** wordt onderzoek op het gebied van tryptofaan-gerelateerde componenten (indolen) beschreven. Deze endogene stoffen spelen een belangrijke rol in verscheidene (patho)fysiologische processen. Overmatige serotonineproductie is bijvoorbeeld de belangrijkste oorzaak van de endocriene symptomen die kunnen optreden bij gemetastaseerde carcinoid tumoren. Verlaagde tryptofaan en serotonineconcentraties in biologische matrices zijn betrokken bij verschillende psychiatrische ziekten, zoals depressie. Een verlaagde tryptofaan beschikbaarheid en de vorming van neurotoxische intermediairen, zoals quinoline zuur, treden op als gevolg van immuunactivatie. Dit wordt veroorzaakt door inductie van enzymatische omzetting van tryptofaan naar kynurenine metabolieten. De metabole route die tryptofaan volgt, is afhankelijk van factoren zoals de tryptofaan beschikbaarheid en enzymactiviteiten. Deze factoren beïnvloeden de mate van synthese van de tryptofaan-afgeleide indolen, zoals serotonine en melatonine.

Serotonine-producerende gemetastaseerde carcinoide tumoren bevinden zich primair meestal in de dunne darm. Indien gemetastaseerd kunnen deze tumoren aanleiding geven tot een carcinoidsyndroom met symptomen zoals opvliegers, diarree, afwijkingen in de rechterkant van het hart en bronchoconstrictie. Een carcinoid tumor kan serotonine synthetiseren vanuit de precursor tryptofaan, waardoor de hoeveelheid beschikbare tryptofaan voor de andere metabole routes afneemt. 5-HIAA in urine is het eindproduct van de serotonine afbraak en de kwantificering van deze metaboliet in urine is de meest gebruikte test voor de follow-up van carcinoid patienten. In **Hoofdstuk 7** beschrijven we de ontwikkeling en klinische validatie van een XLC-MS/MS methode voor deze analyse. Geautomatiseerde opzuivering van de urine gebeurt door middel van interactie met sterk hydrofoob polystereen dragermateriaal, terwijl de chromatografische scheiding ook gebaseerd is op hydrofobe interactie. De detectie vindt plaats met positieve electrosprayionisatie in een quadrupole tandem massaspectrometer. Door de specifieke instellingen duurt de totale analyse inclusief monstervoorbewerking slechts 5 minuten. De absolute opbrengst is 96,5%-99,6% en de analytische variatie is minder dan 5%. De 5-HIAA concentraties in urinemonsters van patiënten met gemetastaseerde carcinoiden verkregen met XLC-MS/MS correleren uitstekend met de voormalige HPLC-fluorometrische methode (R^2=0.99). Dus, de XLC–MS/MS methode beschreven in hoofdstuk 7 laat zien dat de ontwikkelde routinebepaling van 5-HIAA in urine een korte analysetijd heeft en een hoge precisie, gevoeligheid en specificiteit.

In **Hoofdstuk 8A** is een XLC-MS/MS methode ontwikkeld voor de analyse van serotonine in urine die de voormalige conventionele HPLC techniek met handmatige monstervoorbewerking en fluorometrische detectie vervangt. Serotonine in urine is een marker die soms verhoogd is in het geval van voordarm carcinoid tumoren. Het analytische principe is vergelijkbaar met dat gebruikt voor de bepaling van de metanefrinen zoals beschreven in Hoofdstuk 3. Hiervoor wordt een zwakke kation uitwisseling extractie als monstervoorbereiding en hydrofiele interactie chromatografie voor scheiding toegepast. Detectie vindt plaats met een quadrupole tandem massaspectrometer met positieve electrosprayionisatie. Door de specifieke instellingen is de totale analysetijd 6 minuten, inclusief monstervoorbewerking, met een uitstekende lineariteit van 30-7300 µmol/L en een analytische variatie minder dan 10%.
De opgeslagen hoeveelheid serotonine in bloedplaatjes is de meest sensitieve marker voor het aantonen van serotonine overproductie en is daarom een belangrijke marker voor de biochemische diagnose van (gemetastaseerde) carcinoiden. De vrije serotoninefractie in het bloed is de actieve fractie en het is waarschijnlijk die fractie die, indien verhoogd, schade in het lichaam kan aanrichten. In hoofdstuk 8 beschrijven we hoe vrij serotonine gemeten kan

worden in plaatjes-arm plasma. We ontwikkelden hiervoor een specifieke en sensitieve XLC-MS/MS methode met een kwantificeringslimiet van 0,9 nmol/L. Gebruikmakend van deze methode laten we zien, dat de vrije serotonineconcentratie in plasma van gemetastaseerde carcinoid patiënten gemiddeld 13 keer hoger ligt dan in plasma van gezonde controles. De kwantificering van vrij serotonine in plaatjes-arm plasma kan waardevol zijn voor toekomstig onderzoek naar de rol van vrij serotonine. Plasma (vrij) serotonine lijkt in diermodellen een belangrijke rol te spelen in de ontwikkeling van serotonine heropname remmers (SSRI)-afhankelijke osteoporose. In **Hoofdstuk 8B** wordt deze hypothese becommentarieerd en wordt in plasma van gemetastaseerde carcinoidpatienten gemeten hoe hoog de vrije serotonine concentratie is. Het vrije serotonine in plasma is significant hoger in carcinoidpatiënten dan in gezonde controles, terwijl niet bekend is dat deze patiënten meer aan osteoporose leiden. Een mogelijke verklaring voor deze discrepantie is de rol van metabole klaring. SSRI's blokkeren ook de serotoninetransporter in perifere doelwitorganen (bijvoorbeeld botweefsel). Hierdoor ontstaan lokaal verhoogde serotonine concentraties waardoor extra activatie van serotonine-receptoren kan optreden. De gedachte is dat in patiënten met carcinoid de metabole klaring sterk verhoogd is, waardoor lokaal verhoogde receptor activatie wordt voorkomen.

Carcinoid patienten zetten als de tumor veel serotonine produceert het meeste uit de voeding afkomstige tryptofaan om in serotonine. Langdurige verhoging van de serotonine biosynthese kan een afname van de opgeslagen hoeveelheid vrije tryptofaan in het lichaam veroorzaken. Dit zou kunnen leiden tot deficiënties in andere componenten in de tryptofaan metabole route, zoals kynurenine, 3-hydrokynurenine en het eindproduct niacine.
Activatie van het enzym indoleamine deoxygenase (IDO) kan ook leiden tot tryptofaan depletie, omdat dit enzym de enzymatische omzetting van tryptofaan naar kynurenine katalyseert. IDO-expressie neemt met name toe tijdens ontstekingsreacties en infecties. Het doel van **Hoofdstuk 9** is om een XLC-MS/MS methode te ontwikkelen voor de simultane kwantificering van tryptofaan, kynurenine en 3-hydroxykynurenine Er wordt hiervoor gebruik gemaakt van selectieve SPE monstervoorbewerking die berust op een sterke kation uitwisselings extractie. Chromatografische scheiding wordt verkregen door hydrofobe interactie chromatografie. Detectie vindt plaats in een quadrupole tandem massaspectrometer met positieve electrosprayionisatie. Door de specifieke instellingen is de totale analysetijd 8 minuten, inclusief monstervoorbewerking. Het minimaal vereiste plasmavolume is teruggebracht tot 10 µL, waardoor ook de analyse van (bloed)monsters van muizen en ratten mogelijk is. De analytische variatie is minder dan 9% en detectiegrenzen zijn 30 nmol/L voor tryptofaan, 1 nmol/L voor kynurenine en 5 nmol/L voor 3-hydroxykynurenine. Referentiewaarden zijn vergelijkbaar met eerder gepubliceerde

waarden. Bloedafname voor tryptofaan en tryptofaan-kynurenine-ratio bepaling dient, vanwege de biologische variatie gedurende de dag, bij voorkeur in nuchtere toestand plaats te vinden.

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Helma

List of Publications

1. de Jong WHA, Graham KS, van der Molen JC, Links TP, Morris MR, Ross HA, de Vries EGE, Kema IP. Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry. Clin Chem 2007;53:1684-93.

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8. de Jong WHA, Wilkens MHLI, de Vries EGE, Kema, IP. Automated mass spectrometric analysis of urinary and plasma serotonin. Anal Bioanal Chem; in press.

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10. de Jong WHA, Post WJ, Kerstens MN, de Vries EGE, Kema IP. Elevated urinary free and deconjugated catecholamines after consumption of a catecholamine-rich diet. Revised and resubmitted.

11. de Jong WHA, de Vries EGE, Kema IP. Current status and future developments of LC-MS/MS in clinical chemistry for quantification of neurochemical biomarkers. Submitted.

Curriculum vitae

Wilhelmina Hiltje Agnes (Helma) de Jong is op 27 april 1982 geboren in Oldebroek. Na het cum laude behalen van haar diploma aan het Gymnasium Celeanum te Zwolle in 2000, verhuisde zij naar Groningen voor de studie farmaceutische wetenschappen. In 2003 werd het Bachelor diploma cum laude behaald, gevolgd door het Master diploma Medisch Farmaceutische Wetenschappen in 2005. Tijdens de studie was zij gedurende twee jaar studentassistente bij vakken voor eerstejaars farmaciestudenten, te weten practica Analytische Chemie en statistiek werkcolleges. Afstudeeronderzoeken vonden plaats binnen de hematologie en de analytische biochemie.

In juni 2006 is zij gestart met een promotieonderzoek in de klinische chemie bij de afdeling Laboratoriumgeneeskunde in het Universitair Medisch Centrum Groningen, wat heeft geleid tot dit proefschrift in 2010. In december 2009 is zij gestart met de opleiding tot klinisch chemicus in het Universitair Medisch Centrum Groningen.

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